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(54) Title: DETECTION METHOD FOR C-RAF-1 GENES

(57) Abstract

The present invention relates to: (1) a method of identifying an individual at an increased risk for developing cancer, (2) a method for determining a prognosis in patients afflicted with cancer, and (3) a method for determining the proper course of treatment for a patient afflicted with cancer; comprising: amplifying a region of the c-raf-1 gene.

DETECTION METHOD FOR C-RAF-1 GENES

Field of the Invention

10   The present invention relates to (1) a method of identifying an individual at an increased risk for developing cancer, (2) a method for determining a prognosis of patients afflicted with cancer, and (3) a method for determining the proper course of treatment for
15 a patient afflicted with cancer.

Background Information

        Lung cancer claims more lives in the United
20 States than any other neoplasm (R.S. Finley, Am. Pharm.
    NS29, 39 (1989)), and of the various forms lung
    adenocarcinomas have one of the worst prognoses (T.P.
    Miller, Semin. Oncol. 17, 11 (1990)). The incidence of
    adenocarcinoma of the lung (ACL) in the United States is
25 also quickly rising (I. Linnoila, Hematol. Oncol. North.
    Am. 4, 1027 (1990); J.B. Sorensen, H.H. Hansen, Cancer
    Surviv. 8, 671 (1989)). In order to gain insight into
    this complex and deadly disease, a model system for its
    study has been developed. For such a model to provide
30 clinically relevant data several criteria must be met.
    The tumors produced should be histologically equivalent to
    their human counterparts, tumor induction must be reliable
    and reproducible, and the numbers generated must be great
    enough to provide statistical significance. To satisfy
35 these conditions a system has been created which uses two
    inbred mouse strains (NFS/n and AKR) along with

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transplacental exposure to the potent carcinogen N-ethyl-N-nitrosourea (ENU) and promotion with the antioxidant butylated hydroxytoluene (BHT). The resulting tumors were examined for altered expression or structural mutations of
5   genes implicated in lung tumor development such as ras, myc, and raf oncogenes (C.D. Little et al., Nature 306, 194 (1983); P.E. Kiefer et al., Cancer Res., 47, 6236 (1987); E. Santos et al., Science 223, 661 (1984); S. Rodenhuis, N. Engl. J. Med. 317, 929 (1987); M. Barbacid,
10   Eur. J. Clin. Invest. 20, 225 (1990); U.R. Rapp et al., J. Int. Assoc. for the Study of Lung Cancer 4, 162 (1988); M.J. Birrer et al., Ann. Rev. Med. 40, 305 (1989); G. Sithanandam et al., Oncogene 4, 451 (1989)).

15   raf proto-oncogenes are evolutionarily highly conserved genes encoding cytoplasmic serine/threonine specific kinases, which function in mitogen signal transduction (reviewed in U.R. Rapp et al., The Oncogene Handbook, T. Curran et al., Eds. (Elsevier Science
20   Publishers, The Netherlands, 1988), pp. 115-154; U.R. Rapp, Oncogene 6, 495 (1991)). The three known active members in the raf family encode phosphoproteins of similar size (72/74 kD for Raf-1; 68 kD for A-Raf-1, and 74 kD for B-Raf (U.R. Rapp et al., in Retroviruses and
25   Human Pathology, R. Gallo et al., Eds. (Humana Press, Clifton, New Jersey 1985), pp. 449-472; T.W. Beck et al., Nucleic Acids Res. 15, 595 (1987); G. Sithanandam et al., Oncogene 5, 1775 (1990))). Raf-1 was first identified as the cellular homologue of v-raf (H.W. Jansen et al.,
30   Nature 307, 218 (1984)), the transforming gene of 3611 MSV (U.R. Rapp et al., J. Virol. 45, 914 (1983); U.R. Rapp et al., Proc. Natl. Acad. Sci. USA 80, 4218 (1983)). Amino acid comparisons of raf family genes shows three conserved regions [CR1, CR2, CR3] (T.W. Beck et al., Nucleic Acids
35   Res. 15, 595 (1987)); CR1 is a regulatory region surrounding a Cys finger consensus sequence, CR2 is a serine/threonine rich region, and CR3 represents the

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kinase domain. Raf-1 has been mapped to chromosome 3p25
in humans (S.J. O'Brien et al., Science 223, 71 (1984)),
and this region has been found to be frequently altered in
small cell lung carcinoma (SCLC) (J. Whang-Peng et al.,
5   Cancer Genet. Cytogenet. 6, 119 (1982); J.M. Ibson et al.,
J. Cell. Biochem. 33, 267 (1987)), familial renal cell
carcinoma (A.J. Cohen et al., N. Engl. J. Med. 301, 592
(1979); G. Kovacs et al., Int. J. Cancer 40, 171 (1987)),
mixed parotid gland tumors (J. Mark et al., Hereditas 96,
10 141 (1982)), and ovarian cancer (K. Tanaka et al., Cancer
Genet. Cytogenet. 43, 1 (1989)).

        Raf genes are differentially expressed in
various tissues (S.M. Storm et al., Oncogene 5, 345
15 (1990)). c-raf-1 has been found to be expressed
ubiquitously, though absolute levels vary between tissues.
A-raf-1 is present predominantly in the urogenital
tissues, whereas B-Raf is most abundant in cerebrum and
testis. The ubiquitous c-Raf-1 kinase is regulated by
20 tyrosine and serine phosphorylations that result from
activated growth factor receptor kinases (D.K. Morrison et
al., Cell 58, 648 (1989); D.K. Morrison et al., Proc.
Natl. Acad. Sci. USA 85, 8855 (1989); K.S. Kovacina et
al., J. Biol. Chem. 265, 12115 (1990); P.J. Blackshear et
25 al., J. Biol. Chem. 265, 12131 (1990); M.P. Carroll et
al., J. Biol. Chem. 265, 19812 (1990); J.N. Siegel et al.,
J. Biol. Chem. 265, 18472 (1990); B.C. Turner et al.,
Proc. Natl. Acad. Sci. USA 88, 1227 (1991); M. Baccarini
et al., EMBO J. 9, 3649 (1990); H. App et al., Mol. Cell.
30 Biol. 11, 913 (1991)). Raf-1 operates downstream of Ras
in mitogen signal transduction as judged by experiments
using antibody microinjection (M.R. Smith et al., Nature
320, 540 (1986)), c-raf-1 antisense expression constructs
(W. Kolch et al., Nature 349, 426 (1991)), dominant
35 negative mutants (W. Kolch et al., Nature 349, 426
(1991)), and Raf revertant cells. Studies with NIH3T3
cells and brain tissue demonstrated that mitogen treatment

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induces Raf-1 kinase activity and causes a transitory relocation of the active enzyme from the cytoplasm to the nucleus and perinuclear area (Z. Olàh et al., *Exp. Brain. Res.* (in press); U.R. Rapp et al., in *Cold Spring Harbor*

5   *Symposia on Quantitative Biology*, Vol. LIII, Eds. (Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY 1988) pp. 173–184).

Raf-1 coupling has been examined in more than a

10 dozen receptor systems and all strong mitogens stimulated Raf-1 kinase activity (U.R. Rapp, *Oncogene* 6, 495 (1991); D.K. Morrison et al., *Cell* 58, 648 (1989); D.K. Morrison et al., *Proc. Natl. Acad. Sci. USA* 85, 8855 (1989); K.S. Kovacina et al., *J. Biol. Chem.* 265, 12115 (1990); P.J.

15 Blackshear et al., *J. Biol. Chem.* 265, 12131 (1990); M.P. Carroll et al., *J. Biol. Chem.* 265, 19812 (1990); J.N. Siegel et al., *J. Biol. Chem.* 265, 18472 (1990); B.C. Turner et al., *Proc. Natl. Acad. Sci. USA* 88, 1227 (1991); M. Baccarini et al., *EMBO J.* 9, 3649 (1990); H. App et

20 al., *Mol. Cell. Biol.* 11, 913 (1991)), and this stimulation correlated with an increase in Raf-1 phosphorylation leading to a shift in apparent molecular weight.

25   SUMMARY OF THE INVENTION

It is an object of this invention to provide a method of identifying an individual at an increased risk for developing cancer.

30

It is another object of this invention to provide a method for determining a prognosis in patients afflicted with cancer.

35   It is a further object of this invention to provide a method for determining the proper course of treatment for a patient afflicted with cancer.

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Further objects and advantages of the present invention will be clear from the description that follows.

In one embodiment, the present invention relates
5   to a method of identifying an individual at an increased risk for developing cancer, comprising:
        amplifying a region of the c-raf-1 gene;
        analyzing products of the amplification for evidence of mutation; and
10   classifying an individual having one or more mutations in the region as having an increased risk for developing cancer.

In another embodiment, the present invention
15   relates to a method for determining a prognosis in patients afflicted with cancer, comprising:
        amplifying a region of the c-raf-1 gene;
        analyzing products of the amplification for evidence of mutation; and
20   classifying patients having no mutation in said region as being less likely to suffer disease relapse or having an increased chance of survival than those patients having one or more mutations in said region.

25   In a further embodiment, the present invention relates to a method for determining the proper course of treatment for a patient afflicted with cancer, comprising:
        amplifying a region of the c-raf-1 gene;
        analyzing products of said amplification for evidence
30   of mutation;
        identifying a patient having at least one mutation in said region, which patient may require treatment proper for patients having a lesser chance of survival or decreased time to relapse; and
35   identifying a patient lacking mutations in said region, which patients may require treatment proper for

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patients having a greater chance of survival or being less
likely to suffer disease relapse.

BRIEF DESCRIPTION OF THE DRAWINGS

5

Figure 1. Effect of BHT promotion on ENU
tumorigenesis in NFS/n x AKR mice. The X-axis represents
percent tumor induced mortality within each group, and the
Y-axis reflects age in weeks. All animals were exposed to
10 ENU transplacentally at a dose of 0.5mM/Kg of mother's
body weight on day 16 of gestation (presence of vaginal
plug was scored as day one). At two weeks of age mice
were weaned into two separate groups and separated by sex.
Both groups were housed in identical cages and supplied
15 with food (Purina Lab Chow) and water ad libitum.
Beginning at three weeks of age, group 2A (O) was given
weekly intraperitoneal (i.p.) injection of corn oil (0.1
ml), and group 2B (◊) received weekly i.p. injections of
BHT (20 mg/Kg of body weight) dissolved in corn oil.
20 Administration of BHT reduces the mean age of mortality
from approximately 20 weeks to 13, and decreases the
initial age of mortality. These curves are significantly
different (p≤ 0.001) as judged by a 2-tailed Cox test. In
both groups the rate of tumorigenesis was identical for
25 males and females.

Figure 2. Northern blot analysis of proto-
oncogene expression levels in ENU induced tumors.

30   Figure 3. Diagnostic digestion of PCR amplified
Ki-ras genes from ENU induced tumors. Genomic DNA was
isolated from a cesium chloride gradient during RNA
preparations. In each case 10 ng was amplified via PCR
(95°C, 5 min. followed by 35 cycles of 95°C, 1 min. →
35 55°C, 1 min. → 72°, 1 min.) with 2 units of Taq I
polymerase. The primers used (K1; 5'-
AACTTGTGGTGGTTGGACCT-3'→ (SEQ ID NO:6) and K2; <= 3'-

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GTCTTAGTGAAACACCTACT-5' (SEQ ID NO:7)) generate a 79 b.p.
product. The primer K1 ends at codon 12 and contains a
mismatch from normal mouse (Ki-_ras_ sequence its 18th
nucleotide (G → C) creating a BstNI site (CCTGG) in
5   conjunction with a normal codon 12 (GGT). Digestion of
amplified product from a normal allele with BstNI produces
two products of 19 and 60 b.p., whereas a mutation in one
of the first two positions of codon 12 will eliminate the
BstNI site. The presence of two normal alleles results in
10 all of the product being cleaved and the presence of one
mutant and one normal allele will result in only half of
the product cut. In the three panels each sample was run
in duplicate, uncut and cut with BstNI. F1 is DNA from an
untreated NFS/n X AKR F1 mouse, and MCA5 is a murine cell
15 line known to harbor a mutant K-_ras_ codon 12 allele. One
lymphoma (24Ly) and one cell line (117; derived from a
lung adenocarcinoma) display a mutated Ki-_ras_ codon 12
allele; however, 24Ly was a passaged tumor and examination
of the original tumor showed two normal alleles indicating
20 that this mutation was acquired during passaging.

            Figure 4. c-_raf_-1 RNAse protection analysis of
ENU induced tumors. The probe used was a 32P labeled
antisense transcript from the 3' non-coding region of a
25 mouse c-_raf_-1 cDNA to the 3' most StuI site.
Hybridization of this probe with normal RNA results in a
protected fragment of 1.2kb covering the region encoding
the Raf-1 kinase domain. One μg of poly(A)+ RNA from each
tumor and 5 μg of F1 RNA (in order to get comparable
30 signals) was hybridized for 12 hours at 52°C with 200,000
cpm of ^{32}P labeled mouse c-_raf_ antisense transcript.
Hybrids were then digested for 30 minutes with 25 μg RNAse
A and 33 units of RNAse T1 at room temperature. Digested
hybrids were then incubated with 50 μg of proteinase K,
35 phenol/chloroform extracted, ethanol precipitated, and
resuspended in loading dye containing 80% formamide.
Samples were then run on 6% polyacrylamide denaturing

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sequencing gels at 65 watts. Gels were vacuum dried at 80 degrees C and exposed to X-ray film. Probe is undigested probe alone; tRNA is probe hybridized to non-specific RNA; v-raf is probe hybridized to RNA from a v-raf transformed

5 cell line and the bands detected represent single base mismatches between murine c-raf and v-raf; NFS/AKR F1 is probe hybridized with RNA from a normal (untreated) mouse; 24 LY is probe hybridized with RNA from a lymphoma; and the remaining lanes are probe hybridized with RNA isolated

10 from lung tumors.

Figure 5. Schematic of Raf-1 protein showing sites of ENU induced mutations. CR1, CR2, and CR3 represent conserved regions 1, 2 and 3. cDNAs were made

15 from tumor derived poly(A)+ RNA using MoMuLV reverse transcriptase. Primers (MR1 sequence and MR2 sequence) encompassing a 435 base pair region c-raf were then used to amplify this region via PCR. The amplification mixture was then run on 1.7% agarose gels and the 435 bp product

20 isolated. This isolated fragment was then treated with T4 polymerase and cloned into the HincII site of M13mp18 for sequencing. Another set of primers (EMR1 sequence and EMR2 sequence) was designed containing EcoRI sites at the termini and used to amplify a 609 base pair region

25 (encompassing the original 435 base pair region). Isolated products from these reactions were then digested with EcoRI and cloned into the EcoRI site of KS. Sequencing reactions were carried out using the Sequenase kit (USB) according to the recommended protocols for

30 single and double stranded sequencing. Sequencing reactions were run on 6% polyacrylamide denaturing gels at 65 watts. Gels were vacuum dried at 80 degrees C and exposed to X-ray film. In each case a normal allele was also sequenced along with the mutant allele.

35

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Figure 6. Schematic for Identifying c-raf-1
mutations. Primers 1 and 2 are shown in SEQ ID NO:8 and
SEQ ID NO:9, respectively.

5   DETAILED DESCRIPTION OF THE INVENTION

The present invention relates to methods that
involve amplifying a region of the c-raf-1 gene (the
sequence of a mouse c-raf-1 gene is shown in SEQ ID NO:10;
10 the nucleotide and corresponding amino acid sequence of a
human c-raf-1 gene is shown in SEQ ID NO:11 and SEQ ID
NO:12, respectively).

In one embodiment, the present invention relates
15 to a method of identifying an individual at an increased
risk for developing cancer (preferably, lung cancer, T-
cell lymphomas, renal cell carcinoma, ovarian carcinoma,
and mixed parotid gland tumors) comprising: amplifying a
region (preferably by using the polymerase chain reaction
20 method(PCR) or by cloning techniques) of the c-raf-1 gene
of the individual (SEQ ID NO:11)(in one preferred
embodiment, the region encodes amino acids 514 to 535 of
SEQ ID NO:12); analyzing products of the amplification for
evidence of mutation (preferably by DNA sequencing of the
25 region) and classifying an individual having one or more
mutations in the region as having an increased risk for
developing cancer. In one prefered embodiment, the region
encodes amino acids 500 to 550 of SEQ ID NO:12 or amino
acids 450 to 630 of SEQ ID NO:12. In another prefered
30 embodiment, the PCR method employs a primer comprising the
sequence shown in SEQ ID NO:7 and a primer comprising the
sequence shown in SEQ ID NO:8. In another prefered
embodiment, the method comprises the steps shown in Figure
6.
35   In another embodiment, the present invention
relates to a method for determining a prognosis in a
patient afflicted with cancer (preferably, those cancers

listed above). The method comprises: amplifying the
region of the c-raf-1 gene as described above; analyzing
products of the amplification for evidence of mutation as
described above; and classifying a patient having no
5   mutation in the region as being less likely to suffer
disease relapse or having an increased chance of survival
than a patient having one or more mutations in the region.

        In another embodiment, the present invention
10 relates to a method for determining the proper course of
treatment for a patient afflicted with cancer (preferably,
those cancers listed above), comprising: amplifying a
region (described above) of the c-raf-1 gene as described
above; analyzing products of the amplification for
15 evidence of mutation as described above; identifying a
patient having at least one mutation in the region, which
patient may require treatment proper for patients having a
lesser chance of survival or decreased time to relapse;
and identifying a patient lacking mutations in the region,
20 which patients may require treatment proper for patients
having a greater chance of survival or being less likely
to suffer disease relapse.

        Administration of therapeutic agents (cytotoxic
25 or cytostatic) tailored to recognize the mutant Raf-1
protein but not normal Raf-1 could specifically target
tumor cells for death of growth inhibition. Such agents
could be comprised of cytotoxic T-cells, antibodies,
and/or specifically designed chemical compounds.
30
        The following Examples demonstrate consistent
point mutations of the c-raf-1 proto-oncogene, within a
small region of the kinase domain, in a mouse model for
chemical tumor induction. This is the first demonstration
35 of point mutated raf genes in vivo, and the first
isolation of activating in vivo point mutations in the
kinase domain of a proto-oncogene. The tumors examined

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show a selective specificity for Raf-1 mutations as
another family of genes, the ras proto-oncogenes which are
frequently activated by point mutation in both animal and
human tumors (S. Rodenhuis et al., Am. Rev. Respir. Dis.
5 142, S27-30; T.R. Devereux et al., Carcinogenesis 12, 299
(1991)), is not involved.

The present invention is described in further
detail in the following non-limiting examples.
10

EXAMPLES

The following protocols and experimental details
are referenced in the examples that follow:

15 RNA Isolation. Tumors were excised, a small portion
minced in PBS (phosphate buffered saline solution) for
passaging in nude mice, frozen immediately in a dry
ice/ethanol bath, and stored at -70° until RNA extraction.
Frozen tissues were minced on wet ice in a guanidine
20 thiocyanate buffer (4M guanidine thiocyanate 10mM EDTA, 2%
N-lauryl sarcosine, 2% beta-mercaptoethanol, 10mM Tris
(pH=7.6)), disrupted in a Dounce homogenizer, and
extracted three times with phenol: chloroform: isoamyl
alcohol (24:24:2). Supernatants were then transferred to
25 SW41 tubes, 100 µg of cesium chloride per ml was added to
the supernatant which was then underlayed with one half
saturated cesium chloride in 10mM EDTA (pH=7.0; index of
refraction 1.3995-1.4000), and centrifuged at 25,000 rpm
for 20 hours in a Sorvall SW-41TI rotor using a Beckman
30 model L5-50 ultracentrifuge. Supernatants were removed
and RNA pellets dissolved in 4 ml resuspension buffer (10
mM Tris-HCl pH=7.6, 5% beta-mercaptoethanol, 0.5% N-lauryl
sarcosine, 10 mM EDTA), extracted once with
phenol:chloroform:isoamyl alcohol, sodium acetate added to
35 0.12M and RNA precipatated with two volumes ethanol at -
20°C overnight. Precipitates were centrifuged at 9,000 rpm
in a Sorvall SS-34 rotor for 30 minutes, and pellets

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redissolved in RNA sample buffer (10 mM Tris pH=7.4, 1mM
EDTA, 0.05% sodium dodecyl sulfate) and concentrations
determined by absorbance at 260 nm. Poly (A)$^+$ RNA was
isolated by binding to oligo dT cellulose columns in high
5 salt (10mM Tris pH=7.4, 1mM EDTA, 0.05% SDS, 500mM NaCl),
and eluting with RNA sample buffer heated to 40°C.

Northern Blotting. 5 µg poly(A)$^+$ RNA per lane was ethanol
precipitated, desiccated, resuspended in loading buffer
10 (20mM MOPS pH=6.8, 5mM sodium acetate, 1mM EDTA, 50%
formamide, 6% formaldehyde), heated at 65°C for 5 min.,
quick chilled on wet ice for 10 min., and electrophoresed
through a 0.7% agarose gel containing 2.2 M formaldehyde,
20mM MOPS [pH=6.8], 5mM sodium acetate, and 1mM EDTA.
15 Gels were then blotted overnight onto nitrocellulose
filters via capillary transfer in 20X SSC, filters were
washed in 3X SSC for 10 min. and baked at 80°C for 2
hours.

20 Hybridizations. Filters were prehybridized at 42°C in 5X
SSC, 50% formamide, 20mM sodium phosphate pH=6.8, 200
µg/ml PVP-40, 200 µg/ml ficoll 400, 200 µg/ml bovine serum
albumin, and 200 µg/ml sonicated sheared salmon sperm DNA.
Blots were then hybridized with 500,000 cpm/ml of random
25 primed ^{32}P labeled probes overnight at 42°C in
prehybridization solution with 5% dextran sulfate. Blots
were washed with agitation in 2X SSC, 0.1% SDS at room
temperature six times for 20 minutes each wash, then
washed once at 45°C in 0.1X SSC for 15 minutes. Filters
30 were exposed to X-AR 5 film at -70°C.

EXAMPLE 1

Tumor Induction

35   NFS female mice were mated with AKR males and
pregnant females given a transplacental injection of 1-
ethyl-1-nitrosourea (ENU) at a dosage of 0.5 mM/Kg

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mother's body weight on day 16 of gestation, counting plug
date as day one. ENU was chosen for tumor induction since
it is a very potent direct acting carcinogen capable of
modifying any base in vivo (Singer, B. et al., 1983.

5   Molecular Biology of Mutagens and Carcinogens, Plenum
Press, New York). ENU alkylates all tissues with roughly
the same efficiency (E. Scherer et al., Cancer Lett. 46,
21 (1989)) and has a very short half life in vivo (E.M.
Faustman et al., Teratology 40, 199 (1989)) allowing

10 specific mutagenesis of tissues which are mitotically
active at a particular time. NFS and AKR were chosen as
parental strains based on earlier studies which showed
them to be particularly susceptible to ling tumors
following ENU exposure (B.A. Diwan et al., Cancer Res. 34,

15 764 (1974); S.L. Kauffman, JNCI 57, 821 (1976)). With
this procedure nearly 100% of the offspring develop lung
adenocarcinomas and approximately 70% develop, in
addition, T-cell lymphomas with a mean latency of
approximately 20 weeks. In order to achieve more rapid

20 tumor development,weanling mice were treated with weekly
injections of a tumor promoter, the antioxidant butylated
hydroxytoluene or BHT (20mg/kg body weight dissolved in
corn oil). BHT was used as it has been demonstrated to
cause lung lesions and hyperplasia when injected into mice

25 (A.A. Marino et al., Proc. Soc. Exp. Biol. Med. 140, 122
(1972); H. Witschi et al., Proc. Soc. Exp. Biol. Med.,
147, 690 (1974); N. Ito et al., CRC Crit. Rev. Toxicol.
15, 109 (1984)). In the present system it nearly doubles
the rate at which tumors develop. Figure 1 compares tumor

30 induced mortality with age of animals for those receiving
ENU alone, and those receiving ENU and promoted with BHT.
These curves demonstrate that when BHT is given the mean
age of tumor induced mortality decreases from
approximately 20 weeks to around 12, and there is also a

35 decrease in initial latency. These curves are
significantly different with a confidence limit greater
than 99.99% using a 2-tailed Cox test. In addition, BHT

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promotion, while increasing the rate at which tumors
develop, does not affect the tumor spectrum.

EXAMPLE 2

Oncogene Expression

5

Northern blot analysis revealed elevated levels
of c-raf-1, as compared to normal tissue, in every tumor
examined (Figure 2), and Western blot analysis showed that
10 protein levels correlated with message levels (U.R. Rapp
et al., in Oncogenes and Cancer, S.A. Aaronson et al.,
Eds. (Tokyo/VNU Scientific Press, Tokyo, 1987) pp. 55-74).
In addition, in cell lines derived from primary tumors,
Raf-1 protein kinase activity was shown by immune-complex
15 kinase assays to be constitutive. Further analysis of
other oncogenes revealed no consistent pattern of
expression except for ras and myc family genes. In the
case of the myc family, one member (either c-, N-, or L-
myc) was overexpressed but never more than one. For the
20 ras genes, at least one member (Ki-, Ha-, or N-ras), and
often more than one, was expressed at high levels when
compared with the normal tissue. In addition all
oncogenes examined via Northern analysis exhibited full
length, normal sized transcripts.

25

ras genes were considered likely candidates for
mutational activation since oncogenic forms of Ki-ras have
previously been observed in lung tumors (S. Rodenhuis et
al., Am. Rev. Respir. Dis. 142, S27-30; T.R. Devereux et
30 al., Carcinogenesis 12, 299 (1991)) and ENU is a point
mutagen (Singer, B. et al., 1983. Molecular Biology of
Mutagens and Carcinogens, Plenum Press, New York). A
systematic analysis of various ras codons known to be
involved in oncogenic activation was therefore performed .
35 Ha-, Ki-, and N- ras were examined at codons 12, 13, and
61 for potential mutations via RNAse protection assays
(R.M. Myers et al., Science 230, 1242 (1985); E. Winter et

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al., _Proc. Natl. Acad. Sci. USA_ 82, 7575 (1985)), PCR
amplification followed by subsequent sequencing (F. Sanger
et al., _J. Mol. Biol._ 13, 373 (1975)), and PCR
amplification followed by diagnostic restriction digests
5   (W. Jiang et al., _Oncogene_ 4, 923 (1989)). PCR
amplification creating diagnostic enzyme sites is a very
efficient way of examining alleles for mutations at known
sites and involves designing a PCR primer whose 3' end
lies next to and produces a novel restriction site
10 encompassing the codon of interest. Following
amplification, PCR products from normal alleles will
contain the new restriction site, while mutant alleles
will not. Digestion of the product from tissue with two
normal alleles results in all product being cut; however,
15 if one allele contains a mutation, only half of the
product will be digested. Figure 3 shows the results of
amplification and diagnostic digestion applied to Ki-_ras_
codon 12 in several tumors and cell lines. The first
panel is from a set of lymphomas. F1 is DNA from a normal
20 untreated mouse and both alleles are cut by BstN1,
indicating the presence of two normal alleles. MCA5 is a
murine cell line known to contain a Ki-_ras_ codon 12
mutation (L.F. Parada et al., _Mol. Cell. Biol._ 3, 2298
(1983)), and only the amplified normal allele is cleaved.
25 Of the five tumors shown in the second panel, one shows a
mutant Ki-_ras_ allele. The next panel shows some of the
lung tumors tested and none of them exhibit a mutant
allele, and the final panel shows tumor derived cell
lines. The first three are from lymphomas and the last
30 three from lung adenocarcinomas. One lung tumor line
(#117) has a Ki-_ras_ 12 mutation that was not present in
the primary tumor but came up upon transplantation. This
analysis has been performed with Ki, Ha and N-_ras_ genes at
codons 12 and 61. Of all the tumors and cell lines
35 examined by this method for mutations of the three ras
genes at codons 12 and 61, the two shown here were the
only ones detected. Examination of codon 13 was done by

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PCR amplification of genomic DNA surrounding codon 13 followed by cloning into KS+ (Stratagene) and double stranded sequencing. Table I summarizes the ras mutation data. The most notable point from this table is the

5 conspicuous lack of ras mutations in these tumors. In fact the number of ras mutations is much lower than would be expected for a sampling of spontaneous tumors (S. Rodenhuis et al., Am. Rev. Respir. Dis. 142, S27-30; T.R. Devereux et al., Carcinogenesis 12, 299 (1991); J.L. Bos,

10 Cancer Res. 49, 4682 (1989)). Having eliminated ras genes as playing a primary role in the genesis of these ENU induced tumors, c-raf-1 was investigated for possible small or point mutations.

15 TABLE I
Tumors and Cell Lines Positive for ras Mutations

	Codon 12		Codon 13		Codon 61	
	Tumors	Cell Lines	Tumors	Cell Lines	Tumors	Cell Lines
Ha-ras	0/10	0/6	0/6	0/2	0/10	0/6
Ki/ras	1*/10	1/6	0/6	0/2	0/6	0/2
N-ras	0/10	0/6	0/6	0/2	0/10	0/6

* This was a second passage tumor in which the original

25 tumor did not contain a Ki-ras mutation.

Table I: Summary of mutation analysis for Ha-, Ki-, and N-ras at codons 12, 13, and 61. Each box displays the number of mutations detected, over the number of tumors

30 and tumor derived cell lines examined via RNAse protection, sequencing or diagnostic digestion, for each of the nine codons.

EXAMPLE 3
Mutations in Raf-1

35

Since no point mutations had been described for raf genes in vivo, as had been for the ras genes (E.

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Santos et al., Science 223, 661 (1984); S. Rodenhuis, N.
Engl. J. Med. 317, 929 (1987); M. Barbacid, Eur. J.
Clin. Invest. 20, 225 (1990); F. Sanger et al., J. Mol.
Biol. 13, 373 (1975)), point mutations were screened for
5   using RNAse protection assays (R.M. Myers et al.,
Science 230, 1242 (1985); E. Winter et al., Proc. Natl.
Acad. Sci. USA 82, 7575 (1985)). Figure 4 shows a
typical protection assay using a c-raf-1 probe. In this
experiment the probe used covered the 3' end of raf-1
10 from the 3' most StuI site to the end of the coding
sequence. The first lane is a marker (pBR322 digested
with HaeIII), the second shows the probe alone
undigested, the third lane shows the probe hybridized to
unrelated RNA in this case tRNA, the fourth lane shows
15 hybridization with v-raf transformed cells and the lower
bands represent cleavage at points where the mouse c-
raf-1 gene differs from v-raf. The fifth lane shows
hybridization with RNA isolated from a normal lung of an
untreated F1 mouse, the next lanes are RNA isolated from
20 several tumors. In the case of the normal RNA, only
one, fully protected, band is detected while in the case
of the tumors two major bands are seen after digestion.
20 out of 20 tumors analyzed in this fashion showed this
extra band. These data demonstrate the following major
25 points: 1) there is a tumor specific alteration in c-
raf-1 that results in a region of non-homology
recognizable by either RNAse A or T1; 2) The alterations
are confined to the same region of one allele as two
bands of equal size are present in the tumor lanes, and;
30 3) both alleles were expressed at comparable levels as
both bands are of approximately equal intensity. In the
assay shown 5 µg of poly(A)+ RNA was hybridized from
normal tissue, and 1 µg was used from the tumors. This
was necessary to get signals that could be compared on
35 the same gel due to the overexpression of c-raf-1 in the
tumors. By running these assays with various markers it
was possible to estimate the approximate site of the

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alteration(s) to be in the vicinity of the exon 14/exon
15 junction. In order to define the precise genetic
alteration or alterations, PCR primers were designed
which would generate a 600 bp fragment encompassing this
5   region. cDNAs from tumor derived RNA were then
amplified and cloned into KS+ (Stratagene) for double
stranded sequencing. The sequencing results from
several tumors are shown in Figure 5. The top portion
of Figure 5 presents a cartoon of the mouse Raf-1
10   protein. There are three conserved regions CR1, CR2 and
CR3 with CR3 representing the kinase domain. The probe
used in the RNAse protection assays covers the indicated
area, and the PCR primers amplified the bracketed
region. Sequencing through this area revealed a variety
15   of mutations just downstream of the APE site. These
mutants are shown in an expanded version at the bottom
of Figure 5 (See also SEQ ID NO:1 for normal mouse
sequence and SEQ ID NO:2, 3, 4, and 5 for mutant
sequences). These mutants were isolated from four
20   separate tumors, and in each case a normal allele (SEQ
ID NO:1) was also sequenced. Repeating the cDNA
synthesis, PCR amplification, cloning and sequencing
gives the same sequence and normal tissue shows no
mutations demonstrating that these alterations are not
25   artifactual. Sequence covering the amplified region has
been examined and it is interesting that all of these
changes occur within a very small region of the raf
protein. In fact the region where these mutations occur
overlaps an epitope shared by monoclonal antibodies
30   generated against raf (W. Kolch et al., Oncogene 5, 713
(1990)), and computer modeling of the protein shows this
to be a hydrophilic domain, the structure of which is
predicted to be altered by these mutations. This
indicates a biologically important region for the
35   molecule and indeed the first of these mutation tested
in NIH3T3 cell assays, after cloning into a retroviral
expression vector (E1-neo, (G. Heidecker et al., Mol.

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<u>Cell. Biol.</u> 10, 2503 (1990))), was found to be weakly
transforming when driven by a Moloney LTR. The
transformation efficiency was comparable to EC2, a
previously characterized mutation of human c-<u>raf</u>-1 cDNA
5   (G. Heidecker et al., <u>Mol. Cell. Biol.</u> 10, 2503 (1990);
C. Wasylyk et al., <u>Mol. Cell. Biol.</u> 9, 2247 (1989)) and
~20 fold lower than the v-<u>raf</u> oncogene.

                    *   *   *   *   *

10   All publications mentioned hereinabove are
hereby incorporated in their entirety by reference.

            While the foregoing invention has been
described in some detail for purposes of clarity and
15   understanding, it will be appreciated by one skilled in
the art from a reading of this disclosure that various
changes in form and detail can be made without departing
from the true scope of the invention and appended
claims.

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SEQUENCE LISTING

(1) GENERAL INFORMATION:

    (i) APPLICANT: Rapp, Ulf R.
                 Storm, Stephen M.

    (ii) TITLE OF INVENTION: DETECTION METHOD FOR C-RAF-1 GENES

    (iii) NUMBER OF SEQUENCES: 12

    (iv) CORRESPONDENCE ADDRESS:
           (A) ADDRESSEE: NEEDLE & ROSENBERG, P.C.
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           (C) CITY: Atlanta
           (D) STATE: Georgia
           (E) COUNTRY: USA
           (F) ZIP: 30303-1031

    (v) COMPUTER READABLE FORM:
           (A) MEDIUM TYPE: Floppy disk
           (B) COMPUTER: IBM PC compatible
           (C) OPERATING SYSTEM: PC-DOS/MS-DOS
           (D) SOFTWARE: PatentIn Release #1.0, Version #1.25

    (vi) CURRENT APPLICATION DATA:
           (A) APPLICATION NUMBER:
           (B) FILING DATE:
           (C) CLASSIFICATION:

    (viii) ATTORNEY/AGENT INFORMATION:
           (A) NAME: Perryman, David G.
           (B) REGISTRATION NUMBER: 33,438
           (C) REFERENCE/DOCKET NUMBER: 1414.0421

    (ix) TELECOMMUNICATION INFORMATION:
           (A) TELEPHONE: (404) 688-0770
           (B) TELEFAX: (404) 688-9880

(2) INFORMATION FOR SEQ ID NO:1:

    (i) SEQUENCE CHARACTERISTICS:
           (A) LENGTH: 648 amino acids
           (B) TYPE: amino acid
           (D) TOPOLOGY: linear

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

Met Glu His Ile Gln Gly Ala Trp Lys Thr Ile Ser Asn Gly Phe Gly
1   5   10   15

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Leu Lys Asp Ala Val Phe Asp Gly Ser Ser Cys Ile Ser Pro Thr Ile
        20   25   30

Val Gln Gln Phe Gly Tyr Gln Arg Arg Ala Ser Asp Asp Gly Lys Leu
        35   40   45

Thr Asp Ser Ser Lys Thr Ser Asn Thr Ile Arg Val Phe Leu Pro Asn
        50   55   60

Lys Gln Arg Thr Val Val Asn Val Arg Asn Gly Met Ser Leu His Asp
65   70   75   80

Cys Leu Met Lys Ala Leu Lys Val Arg Gly Leu Gln Pro Glu Cys Cys
            85   90   95

Ala Val Phe Arg Leu Leu Gln Glu His Lys Gly Lys Lys Ala Arg Leu
            100   105   110

Asp Trp Asn Thr Asp Ala Ala Ser Leu Ile Gly Glu Glu Leu Gln Val
        115   120   125

Asp Phe Leu Asp His Val Pro Ile Thr Thr His Asn Phe Ala Arg Lys
        130   135   140

Thr Phe Leu Lys Leu Ala Phe Cys Asp Ile Cys Gln Lys Phe Leu Leu
145   150   155   160

Asn Gly Phe Arg Cys Gln Thr Cys Gly Tyr Lys Phe His Glu His Cys
            165   170   175

Ser Thr Lys Val Pro Thr Met Cys Val Asp Trp Ser Asn Ile Arg Gln
            180   185   190

Leu Leu Leu Phe Pro Asn Ser Thr Val Gly Asp Ser Gly Val Pro Ala
        195   200   205

Pro Pro Ser Phe Pro Met Arg Arg Met Arg Glu Ser Val Ser Arg Met
        210   215   220

Pro Ala Ser Ser Gln His Arg Tyr Ser Thr Pro His Ala Phe Thr Phe
225   230   235   240

Asn Thr Ser Ser Pro Ser Ser Glu Gly Ser Leu Ser Gln Arg Gln Arg
            245   250   255

Ser Thr Ser Thr Pro Asn Val His Met Val Ser Thr Thr Leu His Val
            260   265   270

Asp Ser Arg Met Ile Glu Asp Ala Ile Arg Ser His Ser Glu Ser Ala
        275   280   285

Ser Pro Ser Ala Leu Ser Ser Ser Pro Asn Asn Leu Ser Pro Thr Gly
        290   295   300

Trp Ser Gln Pro Lys Thr Pro Val Pro Ala Gln Arg Glu Arg Ala Pro
305   310   315   320

Gly Ser Gly Thr Gln Gln Lys Asn Lys Ile Arg Pro Arg Gly Gln Arg
            325   330   335

Asp Ser Ser Tyr Tyr Trp Glu Ile Glu Ala Ser Glu Val Met Leu Ser
            340   345   350

Thr Arg Ile Gly Ser Gly Ser Phe Gly Thr Val Tyr Lys Gly Lys Trp
        355   360   365

His Gly Asp Val Ala Val Lys Ile Leu Lys Val Val Asp Pro Thr Pro
    370   375   380

Glu Gln Leu Gln Ala Phe Arg Asn Glu Val Ala Val Leu Arg Lys Thr
385   390   395   400

Arg His Val Asn Ile Leu Leu Phe Met Gly Tyr Met Thr Lys Asp Asn
            405   410   415

Leu Ala Ile Val Thr Gln Trp Cys Glu Gly Ser Ser Leu Tyr Lys His
            420   425   430

Leu His Val Gln Glu Thr Lys Phe Gln Met Phe Gln Leu Ile Asp Ile
        435   440   445

Ala Arg Gln Thr Ala Gln Gly Met Asp Tyr Leu His Ala Lys Asn Ile
    450   455   460

Ile His Arg Asp Met Lys Ser Asn Asn Ile Phe Leu His Glu Gly Leu
465   470   475   480

Thr Val Lys Ile Gly Asp Phe Gly Leu Ala Thr Val Lys Ser Arg Trp
            485   490   495

Ser Gly Ser Gln Gln Val Glu Gln Pro Thr Gly Ser Val Leu Trp Met
            500   505   510

Ala Pro Glu Val Ile Arg Met Gln Asp Asp Asn Pro Phe Ser Phe Gln
        515   520   525

Ser Asp Val Tyr Ser Tyr Gly Ile Val Leu Tyr Glu Leu Met Ala Gly
    530   535   540

Glu Leu Pro Tyr Ala His Ile Asn Asn Arg Asp Gln Ile Ile Phe Met
545   550   555   560

Val Gly Arg Gly Tyr Ala Ser Pro Asp Leu Ser Arg Leu Tyr Lys Asn
            565   570   575

Cys Pro Lys Ala Met Lys Arg Leu Val Ala Asp Cys Val Lys Lys Val
            580   585   590

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Lys Glu Glu Arg Pro Leu Phe Pro Gln Ile Leu Ser Ser Ile Glu Leu
    595   600   605

Leu Gln His Ser Leu Pro Lys Ile Asn Arg Ser Ala Ser Glu Pro Ser
    610   615   620

Leu His Arg Ala Ala His Thr Glu Asp Ile Asn Ala Cys Thr Leu Thr
625   630   635   640

Thr Ser Pro Arg Leu Pro Val Phe
                645

(2) INFORMATION FOR SEQ ID NO:2:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 648 amino acids
        (B) TYPE: amino acid
        (D) TOPOLOGY: linear

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

    Met Glu His Ile Gln Gly Ala Trp Lys Thr Ile Ser Asn Gly Phe Gly
    1   5   10   15

    Leu Lys Asp Ala Val Phe Asp Gly Ser Ser Cys Ile Ser Pro Thr Ile
            20   25   30

    Val Gln Gln Phe Gly Tyr Gln Arg Arg Ala Ser Asp Asp Gly Lys Leu
            35   40   45

    Thr Asp Ser Ser Lys Thr Ser Asn Thr Ile Arg Val Phe Leu Pro Asn
        50   55   60

    Lys Gln Arg Thr Val Val Asn Val Arg Asn Gly Met Ser Leu His Asp
    65   70   75   80

    Cys Leu Met Lys Ala Leu Lys Val Arg Gly Leu Gln Pro Glu Cys Cys
                    85   90   95

    Ala Val Phe Arg Leu Leu Gln Glu His Lys Gly Lys Lys Ala Arg Leu
                    100   105   110

    Asp Trp Asn Thr Asp Ala Ala Ser Leu Ile Gly Glu Glu Leu Gln Val
            115   120   125

    Asp Phe Leu Asp His Val Pro Ile Thr Thr His Asn Phe Ala Arg Lys
        130   135   140

    Thr Phe Leu Lys Leu Ala Phe Cys Asp Ile Cys Gln Lys Phe Leu Leu
    145   150   155   160

    Asn Gly Phe Arg Cys Gln Thr Cys Gly Tyr Lys Phe His Glu His Cys
                    165   170   175

Ser Thr Lys Val Pro Thr Met Cys Val Asp Trp Ser Asn Ile Arg Gln
        180   185   190

Leu Leu Leu Phe Pro Asn Ser Thr Val Gly Asp Ser Gly Val Pro Ala
        195   200   205

Pro Pro Ser Phe Pro Met Arg Arg Met Arg Glu Ser Val Ser Arg Met
    210   215   220

Pro Ala Ser Ser Gln His Arg Tyr Ser Thr Pro His Ala Phe Thr Phe
225   230   235   240

Asn Thr Ser Ser Pro Ser Ser Glu Gly Ser Leu Ser Gln Arg Gln Arg
            245   250   255

Ser Thr Ser Thr Pro Asn Val His Met Val Ser Thr Thr Leu His Val
            260   265   270

Asp Ser Arg Met Ile Glu Asp Ala Ile Arg Ser His Ser Glu Ser Ala
        275   280   285

Ser Pro Ser Ala Leu Ser Ser Ser Pro Asn Asn Leu Ser Pro Thr Gly
290   295   300

Trp Ser Gln Pro Lys Thr Pro Val Pro Ala Gln Arg Glu Arg Ala Pro
305   310   315   320

Gly Ser Gly Thr Gln Gln Lys Asn Lys Ile Arg Pro Arg Gly Gln Arg
                325   330   335

Asp Ser Ser Tyr Tyr Trp Glu Ile Glu Ala Ser Glu Val Met Leu Ser
            340   345   350

Thr Arg Ile Gly Ser Gly Ser Phe Gly Thr Val Tyr Lys Gly Lys Trp
        355   360   365

His Gly Asp Val Ala Val Lys Ile Leu Lys Val Val Asp Pro Thr Pro
    370   375   380

Glu Gln Leu Gln Ala Phe Arg Asn Glu Val Ala Val Leu Arg Lys Thr
385   390   395   400

Arg His Val Asn Ile Leu Leu Phe Met Gly Tyr Met Thr Lys Asp Asn
                405   410   415

Leu Ala Ile Val Thr Gln Trp Cys Glu Gly Ser Ser Leu Tyr Lys His
                420   425   430

Leu His Val Gln Glu Thr Lys Phe Gln Met Phe Gln Leu Ile Asp Ile
        435   440   445

Ala Arg Gln Thr Ala Gln Gly Met Asp Tyr Leu His Ala Lys Asn Ile
450   455   460

```
Ile His Arg Asp Met Lys Ser Asn Asn Ile Phe Leu His Glu Gly Leu
465             470             475             480

Thr Val Lys Ile Gly Asp Phe Gly Leu Ala Thr Val Lys Ser Arg Trp
                485             490             495

Ser Gly Ser Gln Gln Val Glu Gln Pro Thr Gly Ser Val Leu Trp Met
            500             505             510

Ala Pro Glu Val Val Arg Met Gln Asp Asp Asn Pro Phe Ser Phe Gln
        515             520             525

Ser Asp Val Tyr Ser Tyr Gly Ile Val Leu Tyr Glu Leu Met Ala Gly
    530             535             540

Glu Leu Pro Tyr Ala His Ile Asn Asn Arg Asp Gln Ile Ile Phe Met
545             550             555             560

Val Gly Arg Gly Tyr Ala Ser Pro Asp Leu Ser Arg Leu Tyr Lys Asn
                565             570             575

Cys Pro Lys Ala Met Lys Arg Leu Val Ala Asp Cys Val Lys Lys Val
            580             585             590

Lys Glu Glu Arg Pro Leu Phe Pro Gln Ile Leu Ser Ser Ile Glu Leu
            595             600             605

Leu Gln His Ser Leu Pro Lys Ile Asn Arg Ser Ala Ser Glu Pro Ser
    610             615             620

Leu His Arg Ala Ala His Thr Glu Asp Ile Asn Ala Cys Thr Leu Thr
625             630             635             640

Thr Ser Pro Arg Leu Pro Val Phe
            645
```

(2) INFORMATION FOR SEQ ID NO:3:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 648 amino acids
        (B) TYPE: amino acid
        (D) TOPOLOGY: linear

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

```
Met Glu His Ile Gln Gly Ala Trp Lys Thr Ile Ser Asn Gly Phe Gly
1               5               10              15

Leu Lys Asp Ala Val Phe Asp Gly Ser Ser Cys Ile Ser Pro Thr Ile
            20              25              30

Val Gln Gln Phe Gly Tyr Gln Arg Arg Ala Ser Asp Asp Gly Lys Leu
    35              40              45
```

—26—

Thr Asp Ser Ser Lys Thr Ser Asn Thr Ile Arg Val Phe Leu Pro Asn
    50   55   60

Lys Gln Arg Thr Val Val Asn Val Arg Asn Gly Met Ser Leu His Asp
65   70   75   80

Cys Leu Met Lys Ala Leu Lys Val Arg Gly Leu Gln Pro Glu Cys Cys
            85   90   95

Ala Val Phe Arg Leu Leu Gln Glu His Lys Gly Lys Lys Ala Arg Leu
        100   105   110

Asp Trp Asn Thr Asp Ala Ala Ser Leu Ile Gly Glu Glu Leu Gln Val
        115   120   125

Asp Phe Leu Asp His Val Pro Ile Thr Thr His Asn Phe Ala Arg Lys
    130   135   140

Thr Phe Leu Lys Leu Ala Phe Cys Asp Ile Cys Gln Lys Phe Leu Leu
145   150   155   160

Asn Gly Phe Arg Cys Gln Thr Cys Gly Tyr Lys Phe His Glu His Cys
            165   170   175

Ser Thr Lys Val Pro Thr Met Cys Val Asp Trp Ser Asn Ile Arg Gln
            180   185   190

Leu Leu Leu Phe Pro Asn Ser Thr Val Gly Asp Ser Gly Val Pro Ala
        195   200   205

Pro Pro Ser Phe Pro Met Arg Arg Met Arg Glu Ser Val Ser Arg Met
    210   215   220

Pro Ala Ser Ser Gln His Arg Tyr Ser Thr Pro His Ala Phe Thr Phe
225   230   235   240

Asn Thr Ser Ser Pro Ser Ser Glu Gly Ser Leu Ser Gln Arg Gln Arg
            245   250   255

Ser Thr Ser Thr Pro Asn Val His Met Val Ser Thr Thr Leu His Val
        260   265   270

Asp Ser Arg Met Ile Glu Asp Ala Ile Arg Ser His Ser Glu Ser Ala
    275   280   285

Ser Pro Ser Ala Leu Ser Ser Ser Pro Asn Asn Leu Ser Pro Thr Gly
290   295   300

Trp Ser Gln Pro Lys Thr Pro Val Pro Ala Gln Arg Glu Arg Ala Pro
305   310   315   320

Gly Ser Gly Thr Gln Gln Lys Asn Lys Ile Arg Pro Arg Gly Gln Arg
            325   330   335

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```
Asp Ser Ser Tyr Tyr Trp Glu Ile Glu Ala Ser Glu Val Met Leu Ser
        340             345             350

Thr Arg Ile Gly Ser Gly Ser Phe Gly Thr Val Tyr Lys Gly Lys Trp
        355             360             365

His Gly Asp Val Ala Val Lys Ile Leu Lys Val Val Asp Pro Thr Pro
    370             375             380

Glu Gln Leu Gln Ala Phe Arg Asn Glu Val Ala Val Leu Arg Lys Thr
385             390             395             400

Arg His Val Asn Ile Leu Leu Phe Met Gly Tyr Met Thr Lys Asp Asn
            405             410             415

Leu Ala Ile Val Thr Gln Trp Cys Glu Gly Ser Ser Leu Tyr Lys His
            420             425             430

Leu His Val Gln Glu Thr Lys Phe Gln Met Phe Gln Leu Ile Asp Ile
        435             440             445

Ala Arg Gln Thr Ala Gln Gly Met Asp Tyr Leu His Ala Lys Asn Ile
    450             455             460

Ile His Arg Asp Met Lys Ser Asn Asn Ile Phe Leu His Glu Gly Leu
465             470             475             480

Thr Val Lys Ile Gly Asp Phe Gly Leu Ala Thr Val Lys Ser Arg Trp
            485             490             495

Ser Gly Ser Gln Gln Val Glu Gln Pro Thr Gly Ser Val Leu Trp Met
        500             505             510

Ala Pro Glu Val Ile Arg Met Gln Asp Asn Asn Pro Phe Ser Phe Gln
        515             520             525

Ser Asp Val Tyr Ser Tyr Gly Ile Val Leu Tyr Glu Leu Met Ala Gly
    530             535             540

Glu Leu Pro Tyr Ala His Ile Asn Asn Arg Asp Gln Ile Ile Phe Met
545             550             555             560

Val Gly Arg Gly Tyr Ala Ser Pro Asp Leu Ser Arg Leu Tyr Lys Asn
            565             570             575

Cys Pro Lys Ala Met Lys Arg Leu Val Ala Asp Cys Val Lys Lys Val
            580             585             590

Lys Glu Glu Arg Pro Leu Phe Pro Gln Ile Leu Ser Ser Ile Glu Leu
        595             600             605

Leu Gln His Ser Leu Pro Lys Ile Asn Arg Ser Ala Ser Glu Pro Ser
    610             615             620
```

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```
Leu His Arg Ala Ala His Thr Glu Asp Ile Asn Ala Cys Thr Leu Thr
625           630              635              640

Thr Ser Pro Arg Leu Pro Val Phe
            645
```

(2) INFORMATION FOR SEQ ID NO:4:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 648 amino acids
        (B) TYPE: amino acid
        (D) TOPOLOGY: linear

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

```
Met Glu His Ile Gln Gly Ala Trp Lys Thr Ile Ser Asn Gly Phe Gly
1               5           10              15

Leu Lys Asp Ala Val Phe Asp Gly Ser Ser Cys Ile Ser Pro Thr Ile
            20              25              30

Val Gln Gln Phe Gly Tyr Gln Arg Arg Ala Ser Asp Asp Gly Lys Leu
            35              40              45

Thr Asp Ser Ser Lys Thr Ser Asn Thr Ile Arg Val Phe Leu Pro Asn
    50              55              60

Lys Gln Arg Thr Val Val Asn Val Arg Asn Gly Met Ser Leu His Asp
65              70              75              80

Cys Leu Met Lys Ala Leu Lys Val Arg Gly Leu Gln Pro Glu Cys Cys
                85              90              95

Ala Val Phe Arg Leu Leu Gln Glu His Lys Gly Lys Lys Ala Arg Leu
            100             105             110

Asp Trp Asn Thr Asp Ala Ala Ser Leu Ile Gly Glu Glu Leu Gln Val
            115             120             125

Asp Phe Leu Asp His Val Pro Ile Thr Thr His Asn Phe Ala Arg Lys
    130             135             140

Thr Phe Leu Lys Leu Ala Phe Cys Asp Ile Cys Gln Lys Phe Leu Leu
145             150             155             160

Asn Gly Phe Arg Cys Gln Thr Cys Gly Tyr Lys Phe His Glu His Cys
                165             170             175

Ser Thr Lys Val Pro Thr Met Cys Val Asp Trp Ser Asn Ile Arg Gln
            180             185             190

Leu Leu Leu Phe Pro Asn Ser Thr Val Gly Asp Ser Gly Val Pro Ala
            195             200             205
```

```
Pro Pro Ser Phe Pro Met Arg Arg Met Arg Glu Ser Val Ser Arg Met
    210             215             220

Pro Ala Ser Ser Gln His Arg Tyr Ser Thr Pro His Ala Phe Thr Phe
225             230             235             240

Asn Thr Ser Ser Pro Ser Ser Glu Gly Ser Leu Ser Gln Arg Gln Arg
                245             250             255

Ser Thr Ser Thr Pro Asn Val His Met Val Ser Thr Thr Leu His Val
            260             265             270

Asp Ser Arg Met Ile Glu Asp Ala Ile Arg Ser His Ser Glu Ser Ala
        275             280             285

Ser Pro Ser Ala Leu Ser Ser Ser Pro Asn Asn Leu Ser Pro Thr Gly
    290             295             300

Trp Ser Gln Pro Lys Thr Pro Val Pro Ala Gln Arg Glu Arg Ala Pro
305             310             315             320

Gly Ser Gly Thr Gln Gln Lys Asn Lys Ile Arg Pro Arg Gly Gln Arg
                325             330             335

Asp Ser Ser Tyr Tyr Trp Glu Ile Glu Ala Ser Glu Val Met Leu Ser
            340             345             350

Thr Arg Ile Gly Ser Gly Ser Phe Gly Thr Val Tyr Lys Gly Lys Trp
        355             360             365

His Gly Asp Val Ala Val Lys Ile Leu Lys Val Val Asp Pro Thr Pro
370             375             380

Glu Gln Leu Gln Ala Phe Arg Asn Glu Val Ala Val Leu Arg Lys Thr
385             390             395             400

Arg His Val Asn Ile Leu Leu Phe Met Gly Tyr Met Thr Lys Asp Asn
            405             410             415

Leu Ala Ile Val Thr Gln Trp Cys Glu Gly Ser Ser Leu Tyr Lys His
            420             425             430

Leu His Val Gln Glu Thr Lys Phe Gln Met Phe Gln Leu Ile Asp Ile
        435             440             445

Ala Arg Gln Thr Ala Gln Gly Met Asp Tyr Leu His Ala Lys Asn Ile
    450             455             460

Ile His Arg Asp Met Lys Ser Asn Asn Ile Phe Leu His Glu Gly Leu
465             470             475             480

Thr Val Lys Ile Gly Asp Phe Gly Leu Ala Thr Val Lys Ser Arg Trp
                485             490             495
```

-30-

```
Ser Gly Ser Gln Gln Val Glu Gln Pro Thr Gly Ser Val Leu Trp Met
            500                 505                 510

Ala Pro Glu Val Ile Arg Met Gln Asp Asp Asn Pro Phe Ser Ser Gln
            515                 520                 525

Ser Asp Val Tyr Ser Tyr Gly Ile Val Leu Tyr Glu Leu Met Ala Gly
    530                 535                 540

Glu Leu Pro Tyr Ala His Ile Asn Asn Arg Asp Gln Ile Ile Phe Met
545                 550                 555                 560

Val Gly Arg Gly Tyr Ala Ser Pro Asp Leu Ser Arg Leu Tyr Lys Asn
                565                 570                 575

Cys Pro Lys Ala Met Lys Arg Leu Val Ala Asp Cys Val Lys Lys Val
            580.                585                 590

Lys Glu Glu Arg Pro Leu Phe Pro Gln Ile Leu Ser Ser Ile Glu Leu
    595                 600                 605

Leu Gln His Ser Leu Pro Lys Ile Asn Arg Ser Ala Ser Glu Pro Ser
    610                 615                 620

Leu His Arg Ala Ala His Thr Glu Asp Ile Asn Ala Cys Thr Leu Thr
625                 630                 ·635                640

Thr Ser Pro Arg Leu Pro Val Phe
                645
```

(2) INFORMATION FOR SEQ ID NO:5:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 648 amino acids
        (B) TYPE: amino acid
        (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

```
Met Glu His Ile Gln Gly Ala Trp Lys Thr Ile Ser Asn Gly Phe Gly
1               5               10                  15

Leu Lys Asp Ala Val Phe Asp Gly Ser Ser Cys Ile Ser Pro Thr Ile
            20                  25                  30

Val Gln Gln Phe Gly Tyr Gln Arg Arg Ala Ser Asp Asp Gly Lys Leu
            35                  40                  45

Thr Asp Ser Ser Lys Thr Ser Asn Thr Ile Arg Val Phe Leu Pro Asn
    50                  55                  60

Lys Gln Arg Thr Val Val Asn Val Arg Asn Gly Met Ser Leu His Asp
65                  70                  75                  80
```

-31-

```
Cys Leu Met Lys Ala Leu Lys Val Arg Gly Leu Gln Pro Glu Cys Cys
                85              90              95

Ala Val Phe Arg Leu Leu Gln Glu His Lys Gly Lys Lys Ala Arg Leu
            100             105             110

Asp Trp Asn Thr Asp Ala Ala Ser Leu Ile Gly Glu Glu Leu Gln Val
            115             120             125

Asp Phe Leu Asp His Val Pro Ile Thr Thr His Asn Phe Ala Arg Lys
    130             135             140

Thr Phe Leu Lys Leu Ala Phe Cys Asp Ile Cys Gln Lys Phe Leu Leu
145             150             155             160

Asn Gly Phe Arg Cys Gln Thr Cys Gly Tyr Lys Phe His Glu His Cys
                165             170             175

Ser Thr Lys Val Pro Thr Met Cys Val Asp Trp Ser Asn Ile Arg Gln
            180             185             190

Leu Leu Leu Phe Pro Asn Ser Thr Val Gly Asp Ser Gly Val Pro Ala
            195             200             205

Pro Pro Ser Phe Pro Met Arg Arg Met Arg Glu Ser Val Ser Arg Met
    210             215             220

Pro Ala Ser Ser Gln His Arg Tyr Ser Thr Pro His Ala Phe Thr Phe
225             230             235             240

Asn Thr Ser Ser Pro Ser Ser Glu Gly Ser Leu Ser Gln Arg Gln Arg
                245             250             255

Ser Thr Ser Thr Pro Asn Val His Met Val Ser Thr Thr Leu His Val
            260             265             270

Asp Ser Arg Met Ile Glu Asp Ala Ile Arg Ser His Ser Glu Ser Ala
        275             280             285

Ser Pro Ser Ala Leu Ser Ser Ser Pro Asn Asn Leu Ser Pro Thr Gly
    290             295             300

Trp Ser Gln Pro Lys Thr Pro Val Pro Ala Gln Arg Glu Arg Ala Pro
305             310             315             320

Gly Ser Gly Thr Gln Gln Lys Asn Lys Ile Arg Pro Arg Gly Gln Arg
            325             330             335

Asp Ser Ser Tyr Tyr Trp Glu Ile Glu Ala Ser Glu Val Met Leu Ser
            340             345             350

Thr Arg Ile Gly Ser Gly Ser Phe Gly Thr Val Tyr Lys Gly Lys Trp
    355             360             365
```

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His Gly Asp Val Ala Val Lys Ile Leu Lys Val Val Asp Pro Thr Pro
    370   375   380

Glu Gln Leu Gln Ala Phe Arg Asn Glu Val Ala Val Leu Arg Lys Thr
385   390   395   400

Arg His Val Asn Ile Leu Leu Phe Met Gly Tyr Met Thr Lys Asp Asn
                405   410   415

Leu Ala Ile Val Thr Gln Trp Cys Glu Gly Ser Ser Leu Tyr Lys His
            420   425   430

Leu His Val Gln Glu Thr Lys Phe Gln Met Phe Gln Leu Ile Asp Ile
        435   440   445

Ala Arg Gln Thr Ala Gln Gly Met Asp Tyr Leu His Ala Lys Asn Ile
    450   455   460

Ile His Arg Asp Met Lys Ser Asn Asn Ile Phe Leu His Glu Gly Leu
465   470   475   480

Thr Val Lys Ile Gly Asp Phe Gly Leu Ala Thr Val Lys Ser Arg Trp
            485   490   495

Ser Gly Ser Gln Gln Val Glu Gln Pro Thr Gly Ser Val Leu Trp Met
        500   505   510

Ala Pro Glu Val Ile Arg Met Gln Asp Asp Asn Pro Phe Ser Phe Gln
    515   520   525

Ser Thr Cys Thr Phe Tyr Gly Ile Val Leu Tyr Glu Leu Met Ala Gly
    530   535   540

Glu Leu Pro Tyr Ala His Ile Asn Asn Arg Asp Gln Ile Ile Phe Met
545   550   555   560

Val Gly Arg Gly Tyr Ala Ser Pro Asp Leu Ser Arg Leu Tyr Lys Asn
            565   570   575

Cys Pro Lys Ala Met Lys Arg Leu Val Ala Asp Cys Val Lys Lys Val
    580   585   590

Lys Glu Glu Arg Pro Leu Phe Pro Gln Ile Leu Ser Ser Ile Glu Leu
    595   600   605

Leu Gln His Ser Leu Pro Lys Ile Asn Arg Ser Ala Ser Glu Pro Ser
    610   615   620

Leu His Arg Ala Ala His Thr Glu Asp Ile Asn Ala Cys Thr Leu Thr
625   630   635   640

Thr Ser Pro Arg Leu Pro Val Phe
            645

(2) INFORMATION FOR SEQ ID NO:6:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 20 base pairs
        (B) TYPE: nucleic acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

AACTTGTGGT GGTTGGACCT   20

(2) INFORMATION FOR SEQ ID NO:7:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 20 base pairs
        (B) TYPE: nucleic acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

TCATCCACAA AGTGATTCTG   20

(2) INFORMATION FOR SEQ ID NO:8:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 20 base pairs
        (B) TYPE: nucleic acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

AGGAGACCAA GTTTCAGATG   20

(2) INFORMATION FOR SEQ ID NO:9:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 20 base pairs
        (B) TYPE: nucleic acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

GCGTGCAAGC ATTGATATCC   20

(2) INFORMATION FOR SEQ ID NO:10:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 1947 base pairs

(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

```
ATGGAGCACA TACAGGGAGC TTGGAAGACG ATCAGCAATG GCTTTGGACT CAAAGATGCG    60

GTGTTTGATG GCTCCAGCTG CATCTCCCCT ACCATTGTTC AGCAGTTTGG CTATCAGCGC   120

CGGGCCTCAG ATGATGGCAA GCTCACGGAT TCTTCTAAGA CAAGCAATAC TATCCGGGTT   180

TTCTTGCCGA ATAAGCAAAG GACTGTGGTC AATGTGCGGA ATGGAATGAG CTTACATGAC   240

TGCCTTATGA AAGCTCTGAA GGTGAGAGGC CTGCAGCCAG AGTGCTGTGC AGTGTTCAGA   300

CTTCTCCAGG AACACAAAGG TAAGAAAGCA CGCTTAGATT GGAACACCGA TGCCGCCTCT   360

CTGATTGGAG AAGAACTGCA AGTGGATTTT TTGGATCATG TTCCCATCAC AACTCACAAC   420

TTTGCTCGGA AAACGTTCCT GAAGCTTGCA TTCTGTGACA TCTGTCAGAA GTTCCTGCTA   480

AATGGATTTC GATGTCAGAC TTGTGGCTAC AAGTTTCATG AGCACTGTAG CACCAAAGTA   540

CCTACTATGT GTGTGGACTG GAGTAATATC AGACAGCTCT TGCTGTTTCC AAATTCCACT   600

GTTGGTGACA GTGGAGTCCC AGCACCACCT TCTTTCCCAA TGCGTCGGAT GCGAGAATCT   660

GTTTCCCGGA TGCCTGCTAG TTCCCAGCAC AGATACTCTA CACCCCATGC CTTCACTTTC   720

AACACCTCCA GCCCTTCCTC AGAAGGTTCC CTCTCCCAGA GGCAGAGGTC AACGTCCACT   780

CCCAATGTCC ACATGGTCAG CACCACCCTG CATGTGGACA GCAGGATGAT TGAGGATGCA   840

ATTCGAAGTC ACAGTGAATC AGCCTCACCT TCAGCCCTGT CCAGCAGCCC AAACAACCTG   900

GGTCCAACAG GCTGGTCACA GCCCAAAACC CCCGTGCCAG CACAAAGAGA GCGGGCACCA   960

GGATCTGGGA CCCAGCAAAA AAACAAAATT AGGCCTCGTG GGCAGAGAGA CTCGAGTTAT  1020

TACTGGGAAA TAGAAGCCAG TGAGGTGATG CTGTCTACTC GGATCGGGTC AGGTTCCTTT  1080

GGCACTGTGT ACAAGGGCAA GTGGCATGGA GATGTTGCAG TAAAGATCCT AAAGGTGGTT  1140

GACCCAACTC CAGAGCAACT TCAGGCCTTC AGGAACGAGG TGGCTGTTTT GCGCAAAACA  1200

CGGCATGTTA ACATCCTGCT GTTCATGGGG TACATGACAA AGGACAACCT GGCGATTGTG  1260

ACTCAGTGGT GTGAAGGCAG CAGTCTCTAC AAACACCTGC ATGTCCAGGA GACCAAATTC  1320

CAGATGTTCC AGCTAATTGA CATTGCCCGA CAGACAGCTC AGGGAATGGA CTATTTGCAT  1380

GCAAAGAACA TCATCCACAG AGACATGAAA TCCAACAATA TATTTCTCCA TGAAGGCCTC  1440

ACGGTGAAAA TTGGAGATTT TGGTTTGGCA ACAGTGAAGT CACGCTGGAG TGGTTCTCAG  1500

CAGGTTGAAC AGCCCACTGG CTCTGTGCTG TGGATGGCCC CAGAAGTAAT CCGGATGCAG  1560
```

```
GATGACAACC CGTTCAGCTT CCAGTCCGAC GTGTACTCGT ACGGCATCGT GCTGTACGAG 1620

CTGATGGCTG GGGAGCTTCC CTACGCCCAC ATCAACAACC GAGACCAGAT CATCTTCATG 1680

GTAGGCCGTG GGTATGCATC CCCTGATCTC AGCAGGCTCT ACAAGAACTG CCCCAAGGCA 1740

ATGAAGAGGT TGGTGGCTGA CTGTGTGAAG AAAGTCAAAG AAGAGAGACC TTTGTTTCCC 1800

CAGATCCTGT CTTCCATCGA GCTGCTTCAG CACTCTCTGC CGAAAATCAA CAGGAGCGCC 1860

TCTGAGCCTT CCCTGCATCG GGCAGCTCAC ACTGAGGACA TCAATGCTTG CACGCTGACT 1920

ACATCCCCAA GGCTACCAGT CTTCTAG .                                   1947
```

(2) INFORMATION FOR SEQ ID NO:11:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 1947 base pairs
        (B) TYPE: nucleic acid
        (C) STRANDEDNESS: double
        (D) TOPOLOGY: linear

    (ix) FEATURE:
        (A) NAME/KEY: CDS
        (B) LOCATION: 1..1944

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

```
ATG GAG CAC ATA CAG GGA GCT TGG AAG ACG ATC AGC AAT GGT TTT GGA    48

Met Glu His Ile Gln Gly Ala Trp Lys Thr Ile Ser Asn Gly Phe Gly
  1               5                  10                  15


TTC AAA GAT GCC GTG TTT GAT GGC TCC AGC TGC ATC TCT CCT ACA ATA    96

Phe Lys Asp Ala Val Phe Asp Gly Ser Ser Cys Ile Ser Pro Thr Ile
             20                  25                  30


GTT CAG CAG TTT GGC TAT CAG CGC CGG GCA TCA GAT GAT GGC AAA CTC   144

Val Gln Gln Phe Gly Tyr Gln Arg Arg Ala Ser Asp Asp Gly Lys Leu
         35                  40                  45


ACA GAT CCT TCT AAG ACA AGC AAC ACT ATC CGT GTT TTC TTG CCG AAC   192

Thr Asp Pro Ser Lys Thr Ser Asn Thr Ile Arg Val Phe Leu Pro Asn
     50                  55                  60


AAG CAA AGA ACA GTG GTC AAT GTG CGA AAT GGA ATG AGC TTG CAT GAC   240

Lys Gln Arg Thr Val Val Asn Val Arg Asn Gly Met Ser Leu His Asp
 65                  70                  75                  80
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```
TGC CTT ATG AAA GCA CTC AAG GTG AGG GGC CTG CAA CCA GAG TGC TGT    288
Cys Leu Met Lys Ala Leu Lys Val Arg Gly Leu Gln Pro Glu Cys Cys
             85          90                    95

GCA GTG TTC AGA CTT CTC CAC GAA CAC AAA GGT AAA AAA GCA CGC TTA    336
Ala Val Phe Arg Leu Leu His Glu His Lys Gly Lys Lys Ala Arg Leu
             100             105             110

GAT TGG AAT ACT GAT GCT GCG TCT TTG ATT GGA GAA GAA CTT CAA GTA    384
Asp Trp Asn Thr Asp Ala Ala Ser Leu Ile Gly Glu Glu Leu Gln Val
             115             120             125

GAT TTC CTG GAT CAT GTT CCC CTC ACA ACA CAC AAC TTT GCT CGG AAG    432
Asp Phe Leu Asp His Val Pro Leu Thr Thr His Asn Phe Ala Arg Lys
             130             135             140

ACG TTC CTG AAG CTT GCC TTC TGT GAC ATC TGT CAG AAA TTC CTG CTC    480
Thr Phe Leu Lys Leu Ala Phe Cys Asp Ile Cys Gln Lys Phe Leu Leu
145             150             155             160

AAT GGA TTT CGA TGT CAG ACT TGT GGC TAC AAA TTT CAT GAG CAC TGT    528
Asn Gly Phe Arg Cys Gln Thr Cys Gly Tyr Lys Phe His Glu His Cys
                 165             170             175

AGC ACC AAA GTA CCT ACT ATG TGT GTG GAC TGG AGT AAC ATC AGA CAA    576
Ser Thr Lys Val Pro Thr Met Cys Val Asp Trp Ser Asn Ile Arg Gln
             180             185             190

CTC TTA TTG TTT CCA AAT TCC ACT ATT GGT GAT AGT GGA GTC CCA GCA    624
Leu Leu Leu Phe Pro Asn Ser Thr Ile Gly Asp Ser Gly Val Pro Ala
             195             200             205

CTA CCT TCT TTG ACT ATG CGT CGT ATG CGA GAG TCT GTT TCC AGG ATG    672
Leu Pro Ser Leu Thr Met Arg Arg Met Arg Glu Ser Val Ser Arg Met
             210             215             220

CCT GTT AGT TCT CAG CAC AGA TAT TCT ACA CCT CAC GCC TTC ACC TTT    720
Pro Val Ser Ser Gln His Arg Tyr Ser Thr Pro His Ala Phe Thr Phe
225             230             235             240

AAC ACC TCC AGT CCC TCA TCT GAA GGT TCC CTC TCC CAG AGG CAG AGG    768
Asn Thr Ser Ser Pro Ser Ser Glu Gly Ser Leu Ser Gln Arg Gln Arg
             245             250             255

TCG ACA TCC ACA CCT AAT GTC CAC ATG GTC AGC ACC ACG CTG CCT GTG    816
```

Ser Thr Ser Thr Pro Asn Val His Met Val Ser Thr Thr Leu Pro Val
            260   265   270

GAC AGC AGG ATG ATT GAG GAT GCA ATT CGA AGT CAC AGC GAA TCA GCC 864

Asp Ser Arg Met Ile Glu Asp Ala Ile Arg Ser His Ser Glu Ser Ala
            275   280   285

TCA CCT TCA GCC CTG TCC AGT AGC CCC AAC AAT CTG AGC CCA ACA GGC 912

Ser Pro Ser Ala Leu Ser Ser Ser Pro Asn Asn Leu Ser Pro Thr Gly
            290   295   300

TGG TCA CAG CCG AAA ACC CCC GTG CCA GCA CAA AGA GAG CGG GCA CCA 960

Trp Ser Gln Pro Lys Thr Pro Val Pro Ala Gln Arg Glu Arg Ala Pro
305   310   315   320

GTA TCT GGG ACC CAG GAG AAA AAC AAA ATT AGG CCT CGT GGA CAG AGA 1008

Val Ser Gly Thr Gln Glu Lys Asn Lys Ile Arg Pro Arg Gly Gln Arg
                325   330   335

GAT TCA AGC TAT TAT TGG GAA ATA GAA GCC AGT GAA GTG ATG CTG TCC 1056

Asp Ser Ser Tyr Tyr Trp Glu Ile Glu Ala Ser Glu Val Met Leu Ser
            340   345   350

ACT CGG ATT GGG TCA GGC TCT TTT GGA ACT GTT TAT AAG GGT AAA TGG 1104

Thr Arg Ile Gly Ser Gly Ser Phe Gly Thr Val Tyr Lys Gly Lys Trp
            355   360   365

CAC GGA GAT GTT GCA GTA AAG ATC CTA AAG GTT GTC GAC CCA ACC CCA 1152

His Gly Asp Val Ala Val Lys Ile Leu Lys Val Val Asp Pro Thr Pro
    370   375   380

GAG CAA TTC CAG GCC TTC AGG AAT GAG GTG GCT GTT CTG CGC AAA ACA 1200

Glu Gln Phe Gln Ala Phe Arg Asn Glu Val Ala Val Leu Arg Lys Thr
385   390   395   400

CGG CAT GTG AAC ATT CTG CTT TTC ATG GGG TAC ATG ACA AAG GAC AAC 1248

Arg His Val Asn Ile Leu Leu Phe Met Gly Tyr Met Thr Lys Asp Asn
                405   410   415

CTG GCA ATT GTG ACC CAG TGG TGC GAG GGC AGC AGC CTC TAC AAA CAC 1296

Leu Ala Ile Val Thr Gln Trp Cys Glu Gly Ser Ser Leu Tyr Lys His
            420   425   430

CTG CAT GTC CAG GAG ACC AAG TTT CAG ATG TTC CAG CTA ATT GAC ATT 1344

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```
Leu His Val Gln Glu Thr Lys Phe Gln Met Phe Gln Leu Ile Asp Ile
        435               440              445

GCC CGG CAG ACG GCT CAG GGA ATG GAC TAT TTG CAT GCA AAG AAC ATC 1392

Ala Arg Gln Thr Ala Gln Gly Met Asp Tyr Leu His Ala Lys Asn Ile
        450           455              460

ATC CAT AGA GAC ATG AAA TCC AAC AAT ATA TTT CTC CAT GAA GGC TTA 1440

Ile His Arg Asp Met Lys Ser Asn Asn Ile Phe Leu His Glu Gly Leu
465               470              475              480

ACA GTG AAA ATT GGA GAT TTT GGT TTG GCA ACA GTA AAG TCA CGC TGG 1488

Thr Val Lys Ile Gly Asp Phe Gly Leu Ala Thr Val Lys Ser Arg Trp
                485              490              495

AGT GGT TCT CAG CAG GTT GAA CAA CCT ACT GGC TCT GTC CTC TGG ATG 1536

Ser Gly Ser Gln Gln Val Glu Gln Pro Thr Gly Ser Val Leu Trp Met
        500               505              510

GCC CCA GAG GTG ATC CGA ATG CAG GAT AAC AAC CCA TTC AGT TTC CAG 1584

Ala Pro Glu Val Ile Arg Met Gln Asp Asn Asn Pro Phe Ser Phe Gln
        515               520              525

TCG GAT GTC TAC TCC TAT GGC ATC GTA TTG TAT GAA CTG ATG ACG GGG 1632

Ser Asp Val Tyr Ser Tyr Gly Ile Val Leu Tyr Glu Leu Met Thr Gly
        530               535              540

GAG CTT CCT TAT TCT CAC ATC AAC AAC CGA GAT CAG ATC ATC TTC ATG 1680

Glu Leu Pro Tyr Ser His Ile Asn Asn Arg Asp Gln Ile Ile Phe Met
545               550              555              560

GTG GGC CGA GGA TAT GCC TCC CCA GAT CTT AGT AAG CTA TAT AAG AAC 1728

Val Gly Arg Gly Tyr Ala Ser Pro Asp Leu Ser Lys Leu Tyr Lys Asn
                565              570              575

TGC CCC AAA GCA ATG AAG AGG CTG GTA GCT GAC TGT GTG AAG AAA GTA 1776

Cys Pro Lys Ala Met Lys Arg Leu Val Ala Asp Cys Val Lys Lys Val
                580              585              590

AAG GAA GAG AGG CCT CTT TTT CCC CAG ATC CTG TCT TCC ATT GAG CTG 1824

Lys Glu Glu Arg Pro Leu Phe Pro Gln Ile Leu Ser Ser Ile Glu Leu
        595               600              605

CTC CAA CAC TCT CTA CCG AAG ATC AAC CGG AGC GCT TCC GAG CCA TCC 1872

Leu Gln His Ser Leu Pro Lys Ile Asn Arg Ser Ala Ser Glu Pro Ser
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	610				615				620					

TTG CAT CGG GCA GCC CAC ACT GAG GAT ATC AAT GCT TGC ACG CTG ACC 1920

Leu His Arg Ala Ala His Thr Glu Asp Ile Asn Ala Cys Thr Leu Thr
625   630   635   640

ACG TCC CCG AGG CTG CCT GTC TTC TAG   1947

Thr Ser Pro Arg Leu Pro Val Phe
              645

(2) INFORMATION FOR SEQ ID NO:12:

        (i) SEQUENCE CHARACTERISTICS:
              (A) LENGTH: 648 amino acids
              (B) TYPE: amino acid
              (D) TOPOLOGY: linear

        (ii) MOLECULE TYPE: protein

        (xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:

Met Glu His Ile Gln Gly Ala Trp Lys Thr Ile Ser Asn Gly Phe Gly
 1   5   10   15

Phe Lys Asp Ala Val Phe Asp Gly Ser Ser Cys Ile Ser Pro Thr Ile
              20   25   30

Val Gln Gln Phe Gly Tyr Gln Arg Arg Ala Ser Asp Asp Gly Lys Leu
              35   40   45

Thr Asp Pro Ser Lys Thr Ser Asn Thr Ile Arg Val Phe Leu Pro Asn
       50   55   60

Lys Gln Arg Thr Val Val Asn Val Arg Asn Gly Met Ser Leu His Asp
 65   70   75   80

Cys Leu Met Lys Ala Leu Lys Val Arg Gly Leu Gln Pro Glu Cys Cys
              85   90   95

Ala Val Phe Arg Leu Leu His Glu His Lys Gly Lys Lys Ala Arg Leu
              100   105   110

Asp Trp Asn Thr Asp Ala Ala Ser Leu Ile Gly Glu Glu Leu Gln Val
              115   120   125

Asp Phe Leu Asp His Val Pro Leu Thr Thr His Asn Phe Ala Arg Lys
       130   135   140

Thr Phe Leu Lys Leu Ala Phe Cys Asp Ile Cys Gln Lys Phe Leu Leu
145   150   155   160

Asn Gly Phe Arg Cys Gln Thr Cys Gly Tyr Lys Phe His Glu His Cys
              165   170   175

Ser Thr Lys Val Pro Thr Met Cys Val Asp Trp Ser Asn Ile Arg Gln
        180   185   190

Leu Leu Leu Phe Pro Asn Ser Thr Ile Gly Asp Ser Gly Val Pro Ala
        195   200   205

Leu Pro Ser Leu Thr Met Arg Arg Met Arg Glu Ser Val Ser Arg Met
    210   215   220

Pro Val Ser Ser Gln His Arg Tyr Ser Thr Pro His Ala Phe Thr Phe
225   230   235   240

Asn Thr Ser Ser Pro Ser Ser Glu Gly Ser Leu Ser Gln Arg Gln Arg
            245   250   255

Ser Thr Ser Thr Pro Asn Val His Met Val Ser Thr Thr Leu Pro Val
        260   265   270

Asp Ser Arg Met Ile Glu Asp Ala Ile Arg Ser His Ser Glu Ser Ala
        275   280   285

Ser Pro Ser Ala Leu Ser Ser Ser Pro Asn Asn Leu Ser Pro Thr Gly
    290   295   300

Trp Ser Gln Pro Lys Thr Pro Val Pro Ala Gln Arg Glu Arg Ala Pro
305   310   315   320

Val Ser Gly Thr Gln Glu Lys Asn Lys Ile Arg Pro Arg Gly Gln Arg
            325   330   335

Asp Ser Ser Tyr Tyr Trp Glu Ile Glu Ala Ser Glu Val Met Leu Ser
            340   345   350

Thr Arg Ile Gly Ser Gly Ser Phe Gly Thr Val Tyr Lys Gly Lys Trp
        355   360   365

His Gly Asp Val Ala Val Lys Ile Leu Lys Val Val Asp Pro Thr Pro
    370   375   380

Glu Gln Phe Gln Ala Phe Arg Asn Glu Val Ala Val Leu Arg Lys Thr
385   390   395   400

Arg His Val Asn Ile Leu Leu Phe Met Gly Tyr Met Thr Lys Asp Asn
            405   410   415

Leu Ala Ile Val Thr Gln Trp Cys Glu Gly Ser Ser Leu Tyr Lys His
        420   425   430

Leu His Val Gln Glu Thr Lys Phe Gln Met Phe Gln Leu Ile Asp Ile
        435   440   445

Ala Arg Gln Thr Ala Gln Gly Met Asp Tyr Leu His Ala Lys Asn Ile
    450   455   460

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```
Ile His Arg Asp Met Lys Ser Asn Asn Ile Phe Leu His Glu Gly Leu
465             470             475         480

Thr Val Lys Ile Gly Asp Phe Gly Leu Ala Thr Val Lys Ser Arg Trp
            485             490             495

Ser Gly Ser Gln Gln Val Glu Gln Pro Thr Gly Ser Val Leu Trp Met
            500             505             510

Ala Pro Glu Val Ile Arg Met Gln Asp Asn Asn Pro Phe Ser Phe Gln
            515             520             525

Ser Asp Val Tyr Ser Tyr Gly Ile Val Leu Tyr Glu Leu Met Thr Gly
            530             535             540

Glu Leu Pro Tyr Ser His Ile Asn Asn Arg Asp Gln Ile Ile Phe Met
545             550             555         560

Val Gly Arg Gly Tyr Ala Ser Pro Asp Leu Ser Lys Leu Tyr Lys Asn
            565             570             575

Cys Pro Lys Ala Met Lys Arg Leu Val Ala Asp Cys Val Lys Lys Val
            580             585             590

Lys Glu Glu Arg Pro Leu Phe Pro Gln Ile Leu Ser Ser Ile Glu Leu
            595             600             605

Leu Gln His Ser Leu Pro Lys Ile Asn Arg Ser Ala Ser Glu Pro Ser
            610             615             620

Leu His Arg Ala Ala His Thr Glu Asp Ile Asn Ala Cys Thr Leu Thr
625             630             635         640

Thr Ser Pro Arg Leu Pro Val Phe
            645
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WHAT IS CLAIMED IS:

1.   A method of identifying an individual at an
increased risk for developing cancer, comprising:
     amplifying a region of the c-raf-1 gene of said
individual;
     analyzing products of said amplification for
evidence of mutation; and
     classifying an individual having one or more
mutations in said region as having an increased risk for
developing cancer.

2.   The method according to claim 1, wherein said
region encodes at least amino acids 514 to 535 of SEQ ID
NO:12.

3.   The method according to claim 2, wherein said
region encodes at least amino acids 500 to 550 of SEQ ID
NO:12.

4.   The method according to claim 3, wherein said
region encodes at least amino acids 450 to 630 of SEQ ID
NO:12.

5.   The method according to claim 1, wherein said
products are analyzed by DNA sequencing.

6.   The method according to claim 1, wherein said
amplification is effected using a polymerase chain
reaction (PCR).

7.   The method according to claim 6, wherein said
PCR employs a primer comprising SEQ ID NO:7 and a primer
comprising SEQ ID NO:8.

8.   A method for determining a prognosis in
patients afflicted with cancer, comprising:

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amplifying a region of the c-raf-1 gene of said
individual;

analyzing products of said amplification for
evidence of mutation; and

classifying a patient having no mutation in said
region as being less likely to suffer disease relapse or
having an increased chance of survival than a patient
having one or more mutations in said region.

9. The method according to claim 8, wherein said
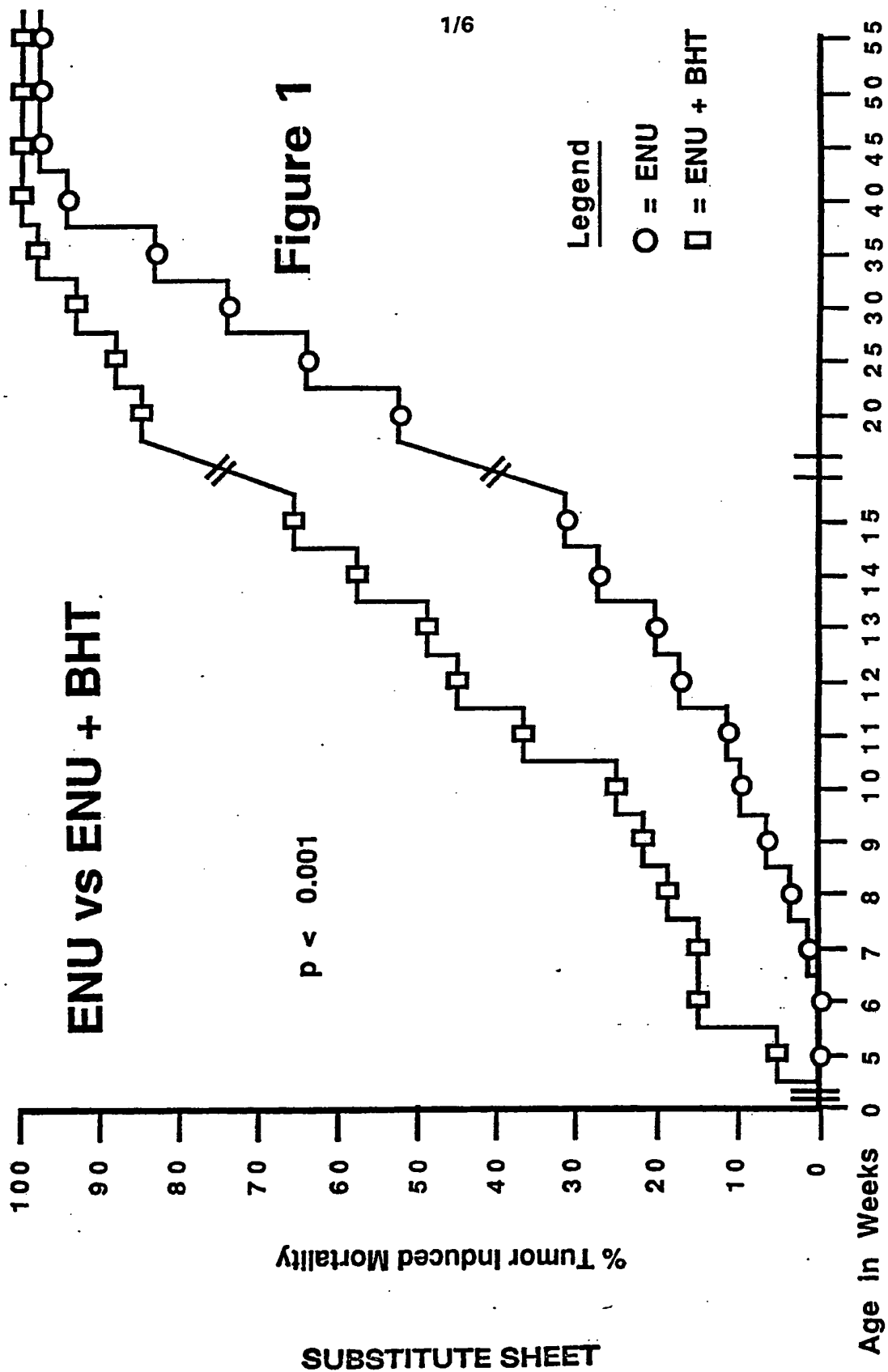region encodes at least amino acids 514 to 535 of SEQ ID
NO:12.

10. The method according to claim 9, wherein said
region encodes at least amino acids 500 to 550 of SEQ ID
NO:12.

11. The method according to claim 10, wherein said
region encodes at least amino acids 450 to 630 of SEQ ID
NO:12.

12. The method according to claim 9, wherein said
products are analyzed by DNA sequencing.

13. The method according to claim 9, wherein said
amplification is effected using polymerase chain
reaction (PCR).

14. The method according to claim 13, wherein said
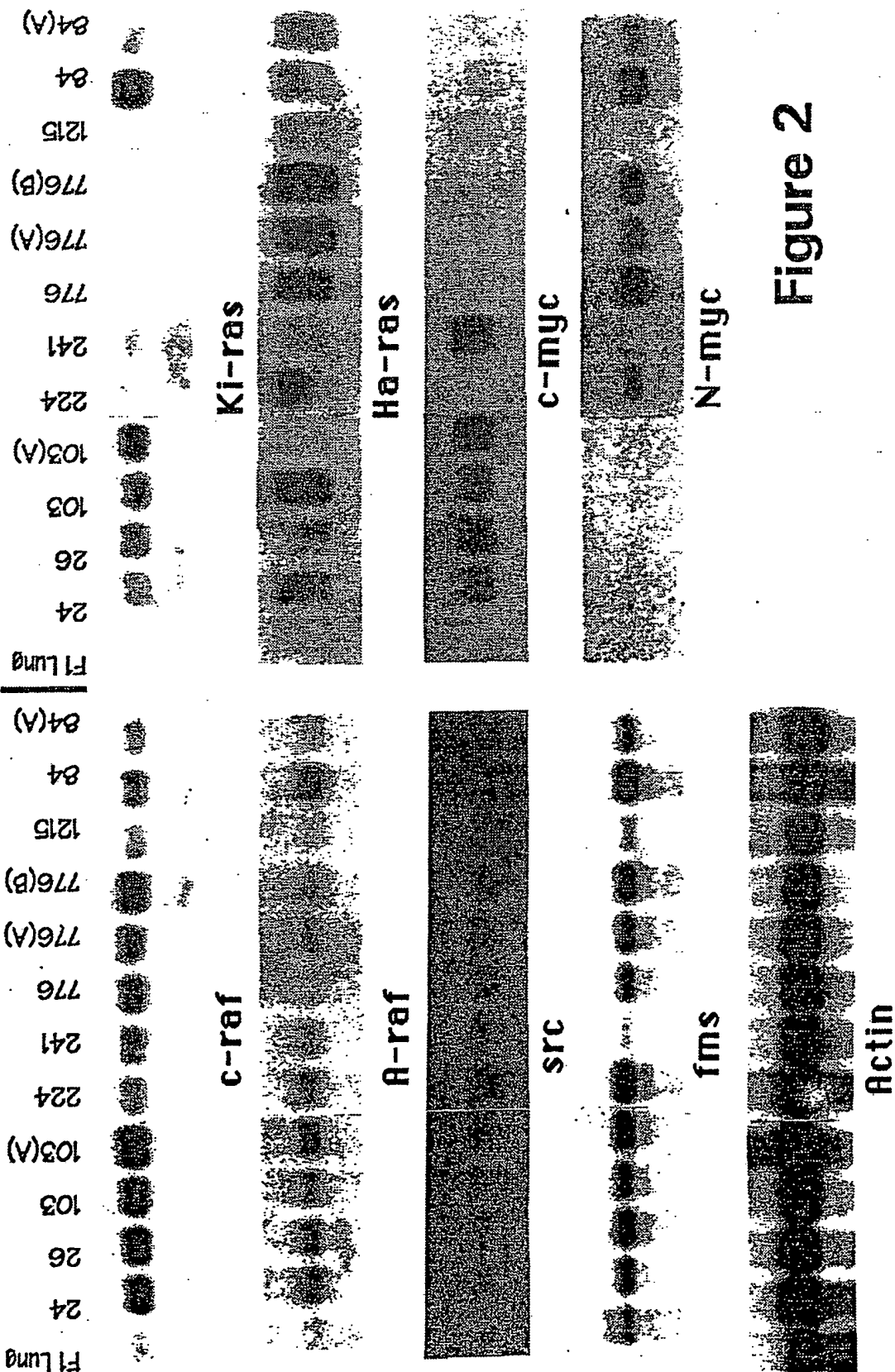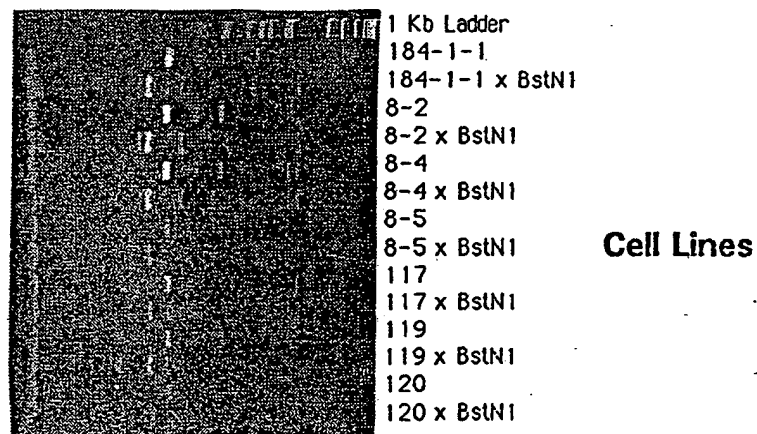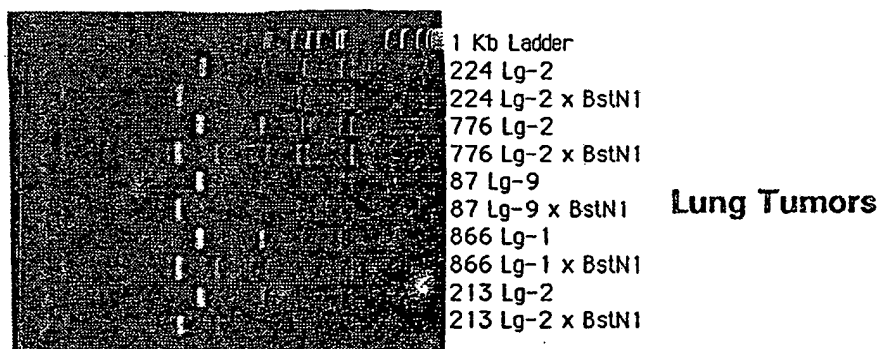PCR employs a primer comprising SEQ ID NO:7 and a primer
comprising SEQ ID NO:8.

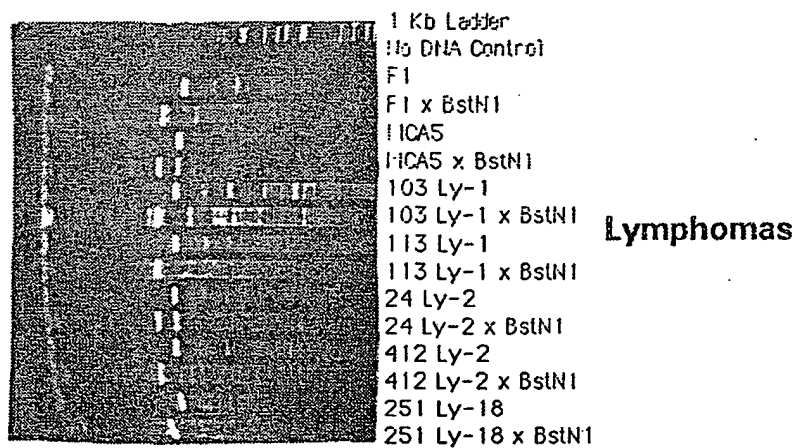15. A method for determining the proper course of
treatment for a patient afflicted with cancer,
comprising:

amplifying a region of the c-raf-1 gene of said
patient;

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analyzing products of said amplification for evidence of mutation;

identifying a patient having at least one mutation in said region, which patient may require treatment proper for a patient having a lesser chance of survival or decreased time to relapse; and

identifying a patient lacking mutations in said region, which patient may require treatment proper for a patient having a greater chance of survival or being less likely to suffer disease relapse.

16. The method according to claim 15, wherein said region encodes at least amino acids 514 to 535 of SEQ ID NO:12.

17. The method according to claim 16, wherein said region encodes at least amino acids 500 to 550 of SEQ ID NO:12.

18. The method according to claim 17, wherein said region encodes at least amino acids 450 to 630 of SEQ ID NO:12.

19. The method according to claim 16, wherein said products are analyzed by DNA sequencing.

20. The method according to claim 16, wherein said amplification is effected using a polymerase chain reaction (PCR).

21. The method according to claim 20, wherein said PCR employs a primer comprising SEQ ID NO:7 and a primer comprising SEQ ID NO:8.

Figure 1

ENU vs ENU + BHT

p < 0.001

Legend

O = ENU
□ = ENU + BHT

Figure 2

Figure 3

1 Kb Ladder
No DNA Control
F1
F1 x BstN1
HCA5
HCA5 x BstN1
103 Ly-1
103 Ly-1 x BstN1   **Lymphomas**
113 Ly-1
113 Ly-1 x BstN1
24 Ly-2
24 Ly-2 x BstN1
412 Ly-2
412 Ly-2 x BstN1
251 Ly-18
251 Ly-18 x BstN1

1 Kb Ladder
224 Lg-2
224 Lg-2 x BstN1
776 Lg-2
776 Lg-2 x BstN1
87 Lg-9
87 Lg-9 x BstN1   **Lung Tumors**
866 Lg-1
866 Lg-1 x BstN1
213 Lg-2
213 Lg-2 x BstN1

1 Kb Ladder
184-1-1
184-1-1 x BstN1
8-2
8-2 x BstN1
8-4
8-4 x BstN1
8-5
8-5 x BstN1   **Cell Lines**
117
117 x BstN1
119
119 x BstN1
120
120 x BstN1

Figure 4

pBR322 + HaeIII

Probe

tRNA

v-raf

NFS/AKR F$_1$

24 LY-1

26 LG-1

103 LG-1

776 LG-2

145 LG-3

776 LG-3

776 LG-3A
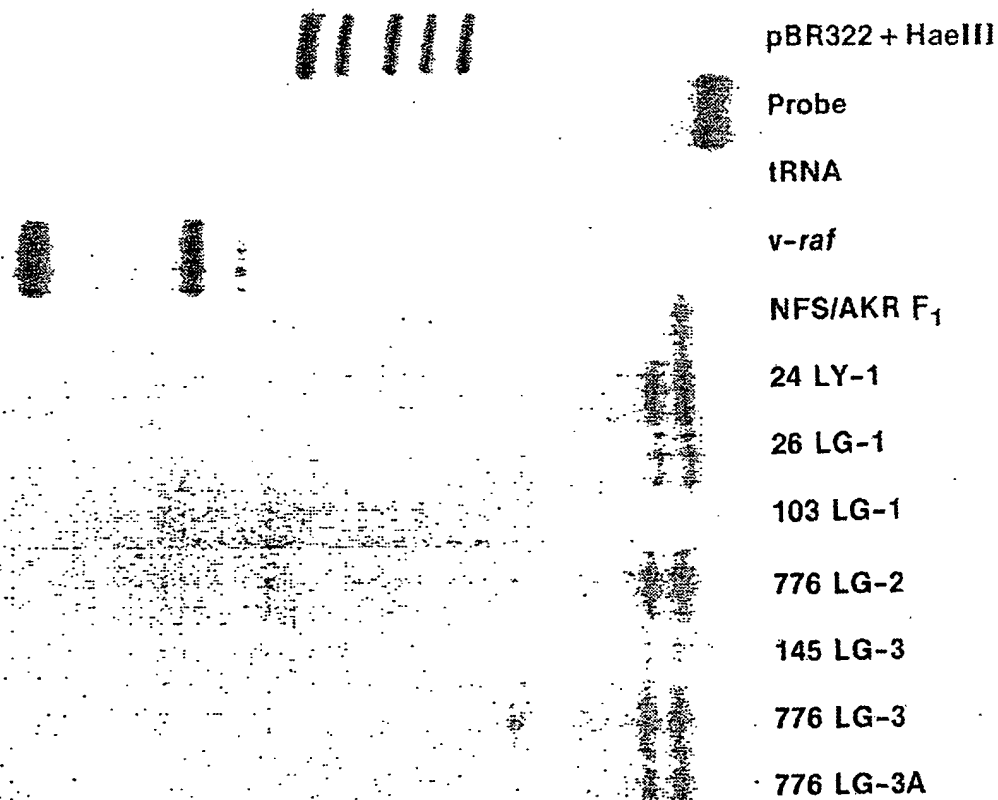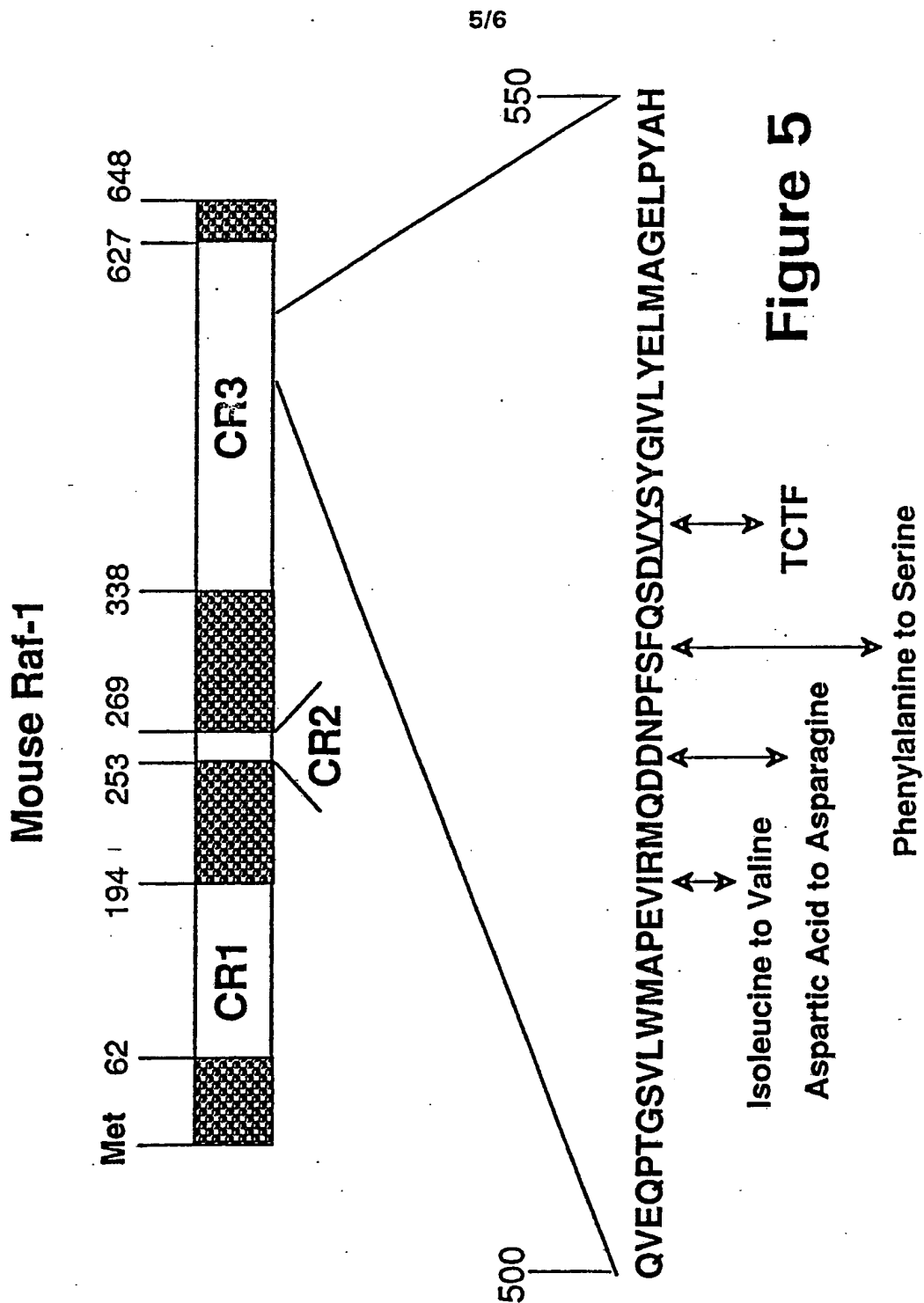
Figure 5

Figure 6

**1. Polymerase Chain Reaction (PCR) Amplification of Target DNA
Either Genomic DNA or cDNA**

Nucleotide 1307 of coding   Nucleotide 1326 of coding

Primer 1 = 5'- AGGAGACCAAGTTTCAGATG -3'

Nucleotide 1915 of coding   Nucleotide 1896 of coding

Primer 2 = 5'- GCGTGCAAGCATTGATATCC -3'

PCR Cycles: 94 ⁰ C, 1 minute

                   55 ⁰ C, 1 minute   **Repeat
                                              35 cycles**

                   72 ⁰ C, 1 minute
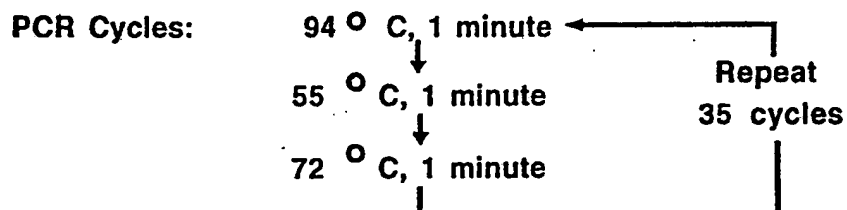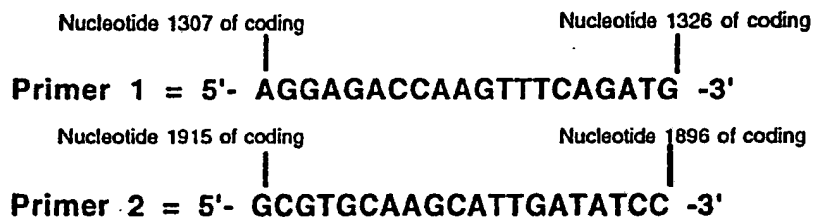
2. Isolate Product

**3. Digestion with
Sphl and Bglll**   **3. Direct Sequencing
                                        Asymmetric PCR**

                                        **5. Standard
                                        Di-Deoxy
                                        Sequencing**

**4. Clone into   4. Clone into
c-raf-1   Alternate
Containing   Vector
Vector**

SUBSTITUTE SHEET

INTERNATIONAL SEARCH REPORT

International Application No. PCT/US 92/07817

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all)[6]
According to International Patent Classification (IPC) or to both National Classification and IPC
IPC5: C 12 Q 1/68

II. FIELDS SEARCHED

Minimum Documentation Searched[7]

Classification System	Classification Symbols
IPC5	C 12 Q

Documentation Searched other than Minimum Documentation
to the Extent that such Documents are Included in Fields Searched[8]

III. DOCUMENTS CONSIDERED TO BE RELEVANT[9]

Category[*]	Citation of Document,[11] with indication, where appropriate, of the relevant passages[12]	Relevant to Claim No.[13]
A	WO, A1, 9112343 (CETUS CORPORATION) 22 August 1991, see the whole document	1-21
A	WO, A1, 9105064 (USA, REPR. BY THE SECR.,D. OF HEALTH/HUMAN S.) 18 April 1991, see the whole document	1-21
A	WO, A1, 9009456 (VIKTOR BALAZS) 23 August 1990, see the whole document	1-21
A	Chemical Abstracts, volume 104, no. 19, 12 May 1986, (Columbus, Ohio, US), Bonner Tom I. et al.: "The complete coding sequence of the human raf oncogene and the corresponding structure of the c-raf-1 gene", see page 141, abstract 162785c, & Nucleic Acids Res. 1986, 14(2), 1009-1015	1-21

* Special categories of cited documents:[10]

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance, the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance, the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
14th December 1992	1 2. 01. 93

International Searching Authority	Signature of Authorized Officer
EUROPEAN PATENT OFFICE	Mikael G:son Bergstrand

Form PCT/ISA/210 (second sheet) (January 1985)

INTERNATIONAL SEARCH REPORT

Box I   Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

> Remark: Claims 1-21
> Diagnostic methods, c.f. PCT Rule 39(iv). Nevertheless, a search
> has been made concerning the subject matter of the application
> (the analytical method).

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II   Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searches without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest   ☐ The additional search fees were accompanied by the applicant's protest.

                              ☐ No protest accompanied the payment of additional search fees.

Form PCT/ISA/210 (continuation of first sheet (1)) (July 1992)

This annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report.
The members are as contained in the European Patent Office EDP file on   02/12/92
The European Patent office is in no way liable for theseparticulars which are merely given for the purpose of information.

Patent document cited in search report	Publication date	Patent family member(s)		Publication date
WO-A1- 9112343	22/08/91	AU-D-	7758991	03/09/91
		EP-A-	0514501	25/11/92
WO-A1- 9105064	18/04/91	AU-D-	6606190	28/04/91
		CA-A-	2067114	03/04/91
		EP-A-	0494968	22/07/92
WO-A1- 9009456	23/08/90	EP-A-	0458831	04/12/91